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Some Properties of the Rabbit Blastocyst

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INTRODUCTION

A CONSIDERABLE, though far from complete, collection of data is available on the chemical composition of the mammalian embryo and foetal fluids in the more advanced stages of pregnancy. But the information on pre-implantation foetal physiology and biochemistry, or the stage immediately succeeding implantation, is still meagre. In the past research on the mammalian blastocyst has depended to a large extent, though not exclusively, upon microscopic methods of study. A notable departure in this respect was created by the investigations initiated by Brambell and his co-workers (Brambell, Hemmings, & Henderson, 1951); based on a combination of immunological and chemical methods, these experiments have provided a new and original approach to problems in this field. The sensitivity of the immunological reactions, coupled with elegant surgical technique, has made it possible to obtain results which have materially widened our understanding, especially of the protein transfer processes between the maternal body and the early embryo.

The present study to which this paper represents an introduction was undertaken in an endeavour (1) to increase our knowledge concerning the chemical composition of the inner milieu of the mammalian blastocyst, and (2) to examine the ability of certain relatively simple chemical substances introduced into the maternal blood-stream to reach the fluid filling the blastocyst cavity, before and immediately after implantation.

MATERIAL AND METHODS

Experimental animals

Sixty-five adult does were used for the experiments; as a routine measure, all were allowed to pass through their first pregnancy and were mated for the actual experiment within a few days after parturition when showing signs of oestrous behaviour. The majority of the experiments were carried out 6, 7, and 8 days after copulation, but on a few occasions foetal fluids were also examined on the 10th and 12th day. A reference hereafter to '6-days-old', '7-days-old', &c., is meant to indicate the interval in days between copulation and the actual experiment.

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In some experiments the does were superovulated by treatment with a gonadotrophin preparation, 'Gonadophysin', presented to the author by Dr. M. C. Chang, whose superovulation procedure (1948) was followed. To obtain the experimental material the animals were first injected intravenously with 0·5 ml./kg. of Nembutal, then thoroughly bled; blood and urine samples were collected in most experiments. The uterus was removed together with the ovarian tubes and ovaries; the number of the corpora lutea was recorded and compared with that of the embryos. On the whole, few discrepancies were noted, except in superovulated does in which the corpora lutea exceeded by 2–3 times the embryos found on the 7th or 8th day. When ovaries were intended for chemical analysis, the fresh luteal tissue was excised and the ovarian tissue containing the follicles was used.

Blastocysts

Six days after mating all blastocysts in our rabbits were found lying free in the uterine cavity, their diameter varying from 1 to 3 mm. Generally, at this stage they were fairly evenly spaced, except in superovulated animals, but quite often their size varied markedly within the same uterine horn.

At 7 days the blastocysts in most of the does appeared implanted, but in a considerable proportion of animals (38 per cent. in this series) the implantation areas were relatively small and the blastocysts were still found lying free, ranging from 3 to 4 mm. in diameter. Actually, at this stage, external inspection usually permitted an approximate differentiation between implanting and non-implanted blastocysts: in the latter case, the implantation sites were not prominent and the blastocysts could be shifted from their positions by gentle pressure upon the uterine wall. The unimplanted blastocysts obtained at 6 and 7 days appeared as transparent colourless spheres; whereas the younger blastocysts were resilient and appeared perfectly round, many of the larger ones removed 1 day later flattened out distinctly when deprived of the support of the uterine wall. The 6-day blastocysts could be handled with some degree of impunity, but the later ones were very delicate and much care had to be devoted to their collection from the uterus preparatory to transfer into chemical glassware.

The surface of the blastocysts appeared to be covered with some material of considerable adhesive power, because when they were placed against the inside of the vertical wall of a glass test-tube they remained firmly in position and did not roll down even if the tube was tilted. It was found that the blastocyst membrane at this stage was remarkably resistant to the action of zinc hydroxide at 100°: when, for the purpose of glucose determination, the blastocysts were placed in a solution of $Zn(OH)_2$ and boiled for 3 minutes, the membrane did not rupture and it required considerable mechanical pressure to pierce it with a pointed glass rod.

The unimplanted blastocysts were lifted from the longitudinally dissected uterus with a small sharp-edged stainless-steel spoon and used whole for chemi-

cal determinations, without rinsing, but after careful wiping of their surface with strips of filter-paper. On the other hand, the fluid filling the blastocyst cavity in implanted blastocysts at 7 and 8 days was collected by puncturing the uterine wall and aspirating with a glass syringe. From the blastocysts at 7 days 0·02–0·05 ml. could usually be collected in this manner per single blastocyst; 1 day later 0·4–0·6 ml. could easily be withdrawn. The fluid which filled the blastocyst cavity at 7 days was colourless, transparent, and did not clot on standing; its pH immediately upon withdrawal was roughly equal to that of maternal plasma, but within a few minutes the pH increased and reached a value of about 8·8. The fluid removed from blastocysts 8 days after mating was faintly yellowish and opalescent, and showed a distinct blue fluorescence in ultra-violet light; it clotted almost at once, owing to the presence at this stage of fibrin (Brambell, 1948). The latter property necessitated prompt pipetting, or the clot was allowed to form and was then broken up mechanically; after it retracted, the fluid portion was used for the experiments. The pH value of the 8-days-old fluid was 7·6–7·8, and did not change appreciably on standing. The difference in pH value and clotting ability between the 7- and 8-days-old blastocyst fluid was so striking that it alone provided a means of distinguishing these two stages of development. Furthermore, whereas upon the addition of trichloroacetic acid or ethanol, no precipitate formed in the 7-day fluid, a heavy precipitate invariably followed the addition of these reagents to the fluid collected one day later from the implanted blastocysts.

On the whole, it was convenient at 7 days to pool the fluid recovered from the blastocysts of any given doe; but on several occasions, especially in superovulated does with numerous embryos, or in others at that stage in which the blastocysts were well developed, sufficient material could be obtained from single blastocysts to permit comparative analyses, e.g. of the glucose content. The amount of fluid available from a single blastocyst a full 8 days after mating usually sufficed for most chemical determinations; otherwise pooled fluid was used. When intact, unimplanted blastocysts were used, they were collected in the bottom part of a conical graduated tube and the volume which they occupied was noted.

Dissection of uterine implantation and interimplantation sites

Several experiments were carried out to determine the glycogen content of the uterus (1) at the level of implantation, (2) in adjacent interimplantation portions, and (3) in the non-pregnant rabbit uterus. For this purpose the fluid was first drained from the blastocysts and next the entire uterine portion containing the implantation site was dissected; similarly, a length of pregnant uterus between two implantation sites, of roughly the same fresh weight, was cut and cleared from adhering fatty tissue; fresh and dry weights were determined. In a limited series of experiments the phosphorus (P) content in such material was also examined.

Alloxan diabetes

To examine the effect of diabetic hyperglycaemia on the glucose level in the blastocyst fluid, diabetes was induced in rabbits by intravenous injection of alloxan, 80 mg./kg. This amount was well tolerated and with the majority of our rabbits resulted in an elevated blood-sugar level and glucosuria within 3–4 days. Some does were first rendered diabetic and then mated for the experiment, but others were mated first and then treated with alloxan on the 3rd or 4th day of pregnancy. Also several superovulated does were given alloxan, which did not appear to interfere with superovulation; as a matter of fact, in one mildly diabetic superovulated doe 30 blastocysts were counted 7 days after mating. In general, however, except where diabetes was slight, the uterus in the alloxan-treated animals appeared thin and poorly vascularized, the embryos were small for their age and seemed to be degenerating.

Administration of sugars and other substances

In these experiments a 50 per cent. solution of sugar (glucose, fructose, sucrose) was injected intravenously, 1·7 g./kg. body weight, into pregnant does 6, 7, and 8 days after copulation; after an interval of 30–60 minutes the animal was nembutalized; blood, blastocysts, urine, and occasionally also ovaries, were used for chemical determinations. In other experiments a solution of Na-sulphapyridine was injected intravenously, 100 mg./kg. body weight. Similarly, the effect of sodium thiocyanate (NaSCN) was examined; a solution of the salt was administered intravenously, or more often subcutaneously, 300 mg./kg. body weight.

Chemical methods

Glucose was determined by the method of Hagedorn & Jensen (1923); the content of 'true' glucose, as distinct from the total reducing value obtained by the Hagedorn & Jensen method, was determined with glucose oxidase (Mann, 1946). Glucose in blastocyst fluid was also identified by paper chromatography, using the method of Trevelyan, Procter, & Harrison (1950). Fructose was determined by the method of Roe (1934); in experiments which involved fructose administration, in addition to fructose glucose was also estimated, both in blood and in the fluid collected from blastocysts. The presence of glucose in urine was detected with the Benedict reagent. Sucrose was determined by carrying out the estimation of reducing sugar in two identical samples, of which one was first hydrolysed for 25 minutes at 100° in the presence of 0·02 N-HCl; the sucrose content was calculated from the difference between these two values. Glycogen estimations were carried out according to Good, Kramer, & Somogyi (1933) using the anthrone reagent (Morris, 1948). Phosphorus was estimated by the method of Fiske & Subbarow (1925). Chloride content was determined according to Whitehorn (1921); nitrogen, potassium, sodium, calcium, and magnesium according to methods described by King (1946) and Pincussen (1928). Sulphapyridine was determined by the method of Bratton & Marshall (1939). Thiocyanate deter-

minations were carried out by the method of Goldstein (1950); 15–20 μg. NaSCN can be accurately estimated by this method.

RESULTS

Dry weight, nitrogen, and phosphorus content of blastocyst fluid

The appearance of the blastocysts and a few general properties of the blastocyst fluid have been remarked upon earlier (p. 3); the dry weight was determined in 2 samples of fluid withdrawn at 7 and 7½ days after mating and in 3 samples at 10 days; the values were 2·5 and 2·2 per cent. and 5·0, 5·3, and 5·8 per cent. respectively, against 7·2 and 7·5 per cent. in rabbit serum. The total N content in fluid aspirated from 7-days-old blastocysts was 30·2–38·5 mg./100 ml. A sample of fluid from the yolk-sac cavity of 10-days-old embryos contained 282·9 mg./100 ml. total N, of which 24·5 mg./100 ml. was non-protein-N; maternal serum contained 845 mg./100 ml. total N and 24·5 mg./100 ml. non-protein-N. The content of phosphate in the pooled fluid from 6 blastocysts collected 7 days after mating was too small to be detected by the chemical method used. But a sample of yolk-sac fluid at 10 days was found to contain 5·6 mg./100 ml. total P, as compared with 10·4 mg./100 ml. in maternal serum.

Chloride, sodium, potassium, magnesium, and calcium content

A determination of chloride was carried out in 7-days-old unimplanted blastocysts, and 390 mg./100 ml. was found. In 2 samples of fluid withdrawn at 10 days 595 and 600 mg./100 ml. were found; at 12 days an estimation gave 640 mg./100 ml.; maternal serum contained 560–595 mg./100 ml. So far the analyses of Na, K, Mg, and Ca have all been carried out in pooled fluid from 10-days-old embryos; the values in mg./100 ml. were: Na 300, K 26·5, Mg. 1·3, Ca 11·2; equivalent values in rabbit serum were: Na 329, K 21·5, Mg. 2·0, Ca 10·2.

Glycogen content of the uterus

The following animals were used for these experiments: 1 pregnant non-superovulated doe, 4 pregnant superovulated does, all 5 animals at 8 days after copulation, and 1 non-pregnant adult doe. The implantation sites in the superovulated animals varied markedly in size, but, in general, those towards the tubal end of the uterus were smaller than the rest; in most non-superovulated animals a similar tendency was evident. The determinations were carried out separately with the small and large implantation sites. Dry-weight determinations on 100 mg. fresh-weight pieces of (1) pregnant uterus, (2) uterus at the level of implantation, and (3) non-pregnant uterus were 17·9, 18·3, and 16·9 mg. respectively.

The findings on glycogen distribution are summarized in Table 1. It can be seen that the size of the individual implantation sites in a given doe varied a great deal. As to the uterine glycogen content, it is likely that the variations found between the animals were to some extent due to breed and age differences.

Concerning the glycogen content of the implantation sites as compared with the interimplantation areas, in some instances, e.g. in does A and C, not much difference was found; but in B there appeared to be a significantly higher concentration in the well-developed implantation sites, as compared both with the poorly developed ones and with the interimplantation areas of the uterus. A relatively high glycogen level in the uterus was found in the two mildly diabetic animals, D and F, of which the latter was non-pregnant.

TABLE 1

Glycogen distribution in the rabbit uterus

(Animals A–E were used 8 days after copulation; all except E were superovulated; E and F were littermates; D and F were mildly diabetic; the blastocysts in D were all small and appeared to be degenerating. The uterus was sectioned transversely; sections at the level of implantation are referred to as 'implant.', those from interimplantation areas as 'interimplant.' The fresh weights and the glycogen values represent means from 2 to 3 determinations.)

Doe (No.)	*Number of embryos*	*Material examined*		*Glycogen mg./100 g. fresh wt.*
		Localization in uterus	*Fresh weight (mg.)*	
A	15	'Implant.'		
		large	280	78
		small	198	56
		'Interimplant.'	—	66
B	17	'Implant.'		
		large	320	128
		small	200	22
		'Interimplant.'	—	48
C	28	'Implant.'		
		large	300	88
		small	205	60
		'Interimplant.'	—	63
D	10	'Implant.'	205	109
		'Interimplant.'	—	108
E	8	'Implant.'		
		large	270	68
		small	218	60
		'Interimplant.'	—	72
F	0	Mid-uterine portion	—	124
		Tubal-end portion	—	110

The content of phosphorus in the uterus

So far three experiments have been carried out in which the content of acid-soluble and total P was determined, at the level of implantation and in interimplantation areas of the uterus, at $7\frac{1}{2}$ days after mating. The mean value in mg./100 g. fresh weight was: acid-soluble P in implantation sites 50, interimplantation sites 83, total P in the former 225, in the latter 329. Much further work is needed to determine the significance of these findings at this early stage of pregnancy.

The content of glucose in blastocyst fluid

In intact unimplanted 6-days-old blastocysts the glucose content as determined by the ferricyanide reduction method of Hagedorn & Jensen was 3–10 mg./100 ml. Determinations carried out with unimplanted blastocysts removed from the uterus 7 days after mating gave values ranging from 18 to 25 mg./100 ml.; but the fluid withdrawn at that stage from what appeared to be already partly implanted blastocysts contained 46–68 mg./100 ml. At 8 days values ranged from 68 to 77 mg./100 ml. In two different samples of 10-days-old yolk-sac fluid the content of total anthrone-reactive carbohydrate was 93·0 and 104 mg./100 ml.; in the same fluids 'true' glucose was determined by incubation with glucose oxidase and a content of 90·0 and 99·2 mg./100 ml., respectively, was found. Another sample of this fluid was examined by paper chromatography; the only sugar detected under these conditions was glucose; fructose and inositol were absent. The glucose content was also examined in the fluid of 8-days-old poorly developed blastocysts from 2 diabetic does which had a blood-sugar level of 247 and 178 mg./100 ml. respectively; however, not more than 82 and 79 mg./100 ml. respectively was found in the blastocyst fluids.

The effect of intravenous administration of glucose, fructose, and sucrose on the sugar content of the blastocyst.

(*a*) *Glucose.* Table 2 gives the results of experiments carried out with does injected with glucose; two of the animals were 6 days and four were 7 days after

TABLE 2

Glucose concentration in blood, blastocysts, and urine following intravenous injection of glucose, 1·7 g./kg. body weight

(In Expts. 1, 2, 7, and 8 whole intact blastocysts were used for determination of glucose, elsewhere blastocyst fluid collected by aspiration was used.)

Experiment (No.)	*Interval after mating (days)*	*Number and condition of blastocysts*	*Interval after injection (min.)*	*Glucose concentration*		
				Blood (mg./100 ml.)	*Blastocyst fluid (mg./100 ml.)*	*Urine (%)*
1	6	9, not implanted	30	283	3	—
2	6	11, not implanted	60	208	7	1·9
3	7	8, implanted	30	413	125	3·0
4	7	5, implanted (?)	30	282	56	2·0
5	7	9, implanted	60	330	209	1·0
6	7	9, implanted	30	317	105	3·0
7	7	7, not implanted	60	300	28	—
8	7	10, not implanted	60	270	22	0·8

copulation; among the latter group, some had blastocysts which were still lying free and were therefore used intact, whereas in the others the uterus showed prominent implantation sites, bulging with fluid which was withdrawn by aspiration. From Expt. Nos. 1 and 2 it can be seen that, regardless of the high

concentration of maternal blood glucose and the concomitant glucosuria, no glucose was found in the fluid present in the 6-days-old blastocysts. Furthermore, insignificant amounts were found in unimplanted blastocysts at 7 days (Expt. Nos. 7 and 8); in these experiments the blastocysts were large enough for every two to provide sufficient material for a single glucose analysis; but results obtained in this manner failed to indicate any appreciable differences in glucose content. The animals in Expt. Nos. 3, 5, and 6 possessed well-developed implantation sites 7 days after mating; 30–60 minutes after the administration of glucose its presence could be unequivocally shown in the fluid withdrawn from the blastocysts. In Expt. No. 4 it was difficult to decide whether or not the blastocysts were already implanted; the concentration of glucose in the aspirated fluid was within the range of values found in implanted blastocysts from untreated animals.

(*b*) *Fructose.* Following intravenous injection of fructose, not only the fructose concentration but also that of glucose was determined as a routine in the maternal blood and, if sufficient material was available, also in the blastocyst fluid. The experiments in Table 3, numbered 1–7, were carried out with animals which had been mated 7 days before; some had unimplanted and others what appeared to be already implanting blastocysts. However, except for a trace in Expt. No. 4, no fructose was detected in the blastocyst fluid at that stage. It was, however, present in the ovarian tissue and in urine.

TABLE 3

Fructose and glucose concentration in blood, blastocysts, ovaries, and urine, following intravenous injection of fructose, 1·7 g./kg. body weight

Experiment. (*No.*)	*Interval after mating* (*days*)	*Number and condition of blastocysts*	*Interval after injection* (*min.*)	*Blood* (*mg./100 ml.*)		*Blastocyst fluid* (*mg./100 ml.*)		*Ovaries* (*mg./100 g.*)	*Urine* (%)
				Fructose	*Glucose*	*Fructose*	*Glucose*	*Fructose*	*Fructose*
1	7	9, implanted	30	204	127	0	100	86	—
2	7	9, implanted	30	130	126	0	60	78	0·8
3	7	11, not implanted	30	130	100	0	29	—	1·1
4	7	6, implanted	45	102	161	trace	130	28	—
5	7	5, not implanted	45	112	173	0	—	46	0·5
6	7	10, not implanted	60	158	107	0	—	—	—
7	7	7, implanted	60	147	138	0	114	45	0·6
8	7½	9, implanted	30	100	79	14	47	32	—
9	7¾	8, implanted	30	159	142	13	59	59	1·2
10	8	7, implanted	30	141	112	35	78	62	1·0
11	8¼	10, implanted	30	150	103	47	88	59	—

On the other hand, already by half a day later, and better still at 8 full days after mating, the presence of fructose could be demonstrated clearly in the blastocyst fluid 30 minutes after fructose administration (Expt. Nos. 8–11). As a matter of fact, the farther advanced the pregnancy, the more the fructose concentration in the blastocyst fluid tended to approach that found in the ovaries.

As is well known, in the rabbit fructose is readily converted into glucose. When the blood of the above animals was analysed, it was found that there was not only considerable fructosaemia but in addition an elevated concentration of glucose; moreover, there was an indication, as in Expt. Nos. 1, 4, and 7, that some of that glucose found its way into the blastocyst fluid, which nevertheless was fructose-free.

(*c*) *Sucrose*. Four experiments have been carried out with intravenous sucrose; the results are presented in Table 4. The amounts of sucrose found in the blastocyst fluid 7 days after mating were so slight as to be insignificant, though possibly in Expt. No. 3 a small amount was present. But 8 days after copulation an appreciable amount of sucrose was found in the blastocyst fluid, as indicated by results based on the determination of the reducing value as well as a positive Seliwanoff reaction.

TABLE 4

Sucrose and glucose concentration in blood and blastocyst fluid, following intravenous injection of sucrose, 1·7 g./kg. body weight

Experiment (No.)	*Interval after mating (days)*	*Number and condition of blastocysts*	*Interval after injection (min.)*	*Blood (mg./100 ml.)*		*Blastocyst fluid (mg./100 ml.)*	
				Sucrose	*Glucose*	*Sucrose*	*Glucose*
1	7	9, implanted	30	310	125	18	68
2	7	9, not implanted	60	207	97	10	35
3	7	5, implanted (?)	60	159	121	37	64
4	8	7, implanted	30	148	117	55	73

TABLE 5

Sulphapyridine concentration in blood, blastocysts, and ovaries, following intravenous injection of Na-*sulphapyridine, 100 mg./kg. body weight*

Experiment (No.)	*Interval after mating (days)*	*Number and condition of blastocysts*	*Interval after injection (min.)*	*Sulphapyridine*		
				Blood (mg./100 ml.)	*Blastocyst fluid (mg./100 ml.)*	*Ovarian tissue (mg./100 g.)*
1	6½	10, not implanted	30	16	2	13
2	7	9, implanted	30	15	trace	—
3	8	5, implanted	30	13	5	7
4	8	9, implanted	60	9	4	5

Effect of sulphapyridine

The results obtained with this substance are shown in Table 5. Following the injection of Na-sulphapyridine, the concentration in blood reached a high level after 30 minutes, but even after an hour a considerable amount still remained in the blood. Unimplanted 6½-days-old blastocysts removed after 30 minutes

from an injected doe were analysed without previous rinsing; a small amount of the sulphonamide drug was detected, but it seems questionable as to whether this was due to its penetration into the blastocysts, or was the result of external surface contamination with sulphapyridine-containing uterine secretion (Expt. No. 1). Ovarian tissue showed a concentration approaching that of blood. Very little sulphapyridine was present in the fluid aspirated from apparently implanted 7-days-old blastocysts (Expt. No. 2). On the other hand, significant amounts were found in fully implanted 8-days-old blastocysts 30 to 60 minutes after the administration of the drug (Expt. Nos. 3 and 4).

Effect of sodium thiocyanate

As can be seen from Table 6, when 300 mg./kg. of this salt was injected intravenously or subcutaneously into rabbits, 30–60 minutes later its presence could be shown in considerable concentration in the maternal blood and in urine. But the fluid collected by aspiration from blastocysts at 7, or even at 8, days after mating contained barely distinguishable amounts of thiocyanate (Expt. Nos. 2, 3, and 4). The fact that some thiocyanate appeared to be present in unimplanted $6\frac{1}{2}$-days-old blastocysts was presumably due to traces of thiocyanate in the uterine secretion adhering to the blastocyst surface (Expt. No. 1).

TABLE 6

Thiocyanate concentration in blood, blastocysts, and urine, following injection of NaCNS, *300 mg./kg. body weight*

Experiment (No.)	*Interval after mating (days)*	*Number and condition of blastocysts*	*Mode of injection*	*Interval after injection (min.)*	NaCNS		
					Blood (mg./100 ml.)	*Blastocyst fluid (mg./100 ml.)*	*Urine (mg./100 ml.)*
1	$6\frac{1}{2}$	10, not implanted	intraven.	30	76	12	52
2	7	5, implanted	subcutan.	30	54	3	—
3	8	7, implanted	subcutan.	30	30	trace (?)	—
4	8	4, implanted	subcutan.	60	53	5	75

DISCUSSION

The main obstacle to an extensive study of the biochemical properties of the early mammalian blastocyst is that, unless unlimited numbers of experimental animals are available, the material for analytical purposes is forthcoming slowly and in rather inadequate quantity. In addition, as in all such work, much individual variation must be anticipated. With that in mind, it was decided to study in the first place the behaviour of substances which could be estimated accurately in small amounts, were well tolerated by the animals, were capable, after parenteral administration, of reaching the maternal blood-stream promptly, and were likely to remain there for a reasonable length of time at a fairly high level of con-

centration. As far as it was practicable, an effort was made to repeat each type of experiment a sufficient number of times to obtain evidence of reproducibility.

The choice of rabbit as experimental animal was guided by the fact that in an oestrous doe ovulation follows copulation with regularity within 10 hours (Walton & Hammond, 1928); this ensured the time of conception within the limits of individual variations of approximately 1 hour. The existence of appreciable individual differences in the progress of early pregnancy was evident, amongst other observations, in the degree of development of blastocysts 7 days after copulation. In some 38 per cent. of the animals in this series the blastocysts at that stage were found lying free in the uterine lumen; but the rest of the animals exhibited well-developed, prominent implantation sites and the blastocysts were presumed to be in the process of incipient implantation. While of course the exact extent of implantation could only be judged accurately by histological methods, it was found that certain simple features of the blastocyst fluid were sufficiently characteristic to allow an approximate estimate of the condition of the blastocysts. Thus, whereas the fluid withdrawn from blastocysts a full 8 days after mating invariably clotted rapidly, contained material precipitable with trichloro-acetic acid or ethanol, and had a pH value of not more than 7·8, the fluid obtained from blastocysts 1 day younger did not clot, became distinctly alkaline on standing, and did not form a precipitate in the presence of protein-precipitating reagents. This behaviour is not surprising in view of the low content of total nitrogen in the fluid at that stage. From experiments still in progress there is evidence of a significant difference in the content of bicarbonate in the blastocyst fluid at 7 and 8 days respectively after copulation.

It is believed that the results of experiments which involve the introduction of simple chemical substances into the maternal blood-stream, and a study of their ability to enter the embryonic fluid within a certain period of time, are likely to form a useful contribution to our knowledge of the extent of contact which is being built up between the maternal organism and the embryo during the early stages of pregnancy. It was interesting to note that, in the rabbit at any rate, simple molecules such as glucose and fructose did not readily penetrate into the unimplanted blastocyst, even when their concentration has reached a high level in the maternal blood and in tissues which form part of the female reproductive tract, e.g. the ovaries. But with incipient implantation, as soon as material links, however tenuous, between the uterine wall and the blastocyst began to form, glucose, though not fructose or sucrose, appeared to be able to pass into the blastocyst fluid. The evidence for this preference for glucose was strengthened by the experiments with fructose administration, in which an increased amount of glucose, but not fructose itself, was found in the blastocyst fluid. Once the process of implantation has been completed, a full 8 days after copulation, all three sugars could be detected within the blastocysts a short time after injection.

The study of the behaviour of these sugars was made easier by the fact that the fluid of the rabbit blastocyst at 6, and even at 7, days after mating contains

relatively little reducing material of its own; there is good evidence that nearly all of it is glucose; fructose and inositol, two carbohydrates known to be present in certain foetal fluids of other species, being absent. It was interesting to observe that the glucose concentration in the blastocyst fluid appeared to be unaffected by maternal hyperglycaemia due to alloxan diabetes. It remains for future experiments to demonstrate to what extent the glucose level in the yolk-sac fluid can be influenced by the action of insulin.

As to the behaviour of parenteral sulphapyridine, while it could be readily detected in the fluid aspirated from fully implanted blastocysts, it is difficult to decide whether the small amount found when whole unimplanted blastocysts were used for analysis indicated its presence inside the blastocyst or, as seems likely, was merely due to contamination of the blastocyst surface with sulphapyridine-containing secretion of the uterine glands. A similar impression was gained in experiments with thiocyanate, so far as early unimplanted blastocysts are concerned; but, unlike the sulphonamide, thiocyanate failed to penetrate in detectable amounts into the fluid of obviously implanted blastocysts, at any rate within the restricted experimental time-interval.

Studies on the distribution of glycogen in the uterus have been hitherto largely concerned with the more advanced stages of pregnancy; as regards the very early stage of pregnancy in the rabbit, which was the object of the present investigation, there was as yet no clear indication of a differential distribution as between the implantation and interimplantation sites of the uterus, although in one experiment at least, distinctly more glycogen was accumulated in the well-developed implantation areas than in the rest of the uterus. Perhaps a clearer picture might emerge if it were possible to limit the investigation to animals of identical age and breed. The suggestion of a significant difference in the pattern of phosphorus distribution in the early pregnant uterus at the level of implantation, as compared with adjacent uterine portions, merits further investigation. Similarly, as more experimental material becomes available it will be interesting to obtain more detailed information of the chemical nature of the fluid which fills the cavity of the 6- and 7-days-old blastocyst, of its inner cell mass, and of the membrane which surrounds it.

SUMMARY

1. A study of certain chemical components of the rabbit blastocyst was carried out using material available at 6, 7, 8, and up to 12 days after copulation. For chemical determinations unimplanted blastocysts were used whole; from the implanted blastocysts the fluid filling the interior was withdrawn by aspiration. The content of nitrogen, chlorides, Na, K, Mg, and Ca was determined. Glucose was identified in yolk-sac fluid and its concentration before and after implantation was determined.

2. The passage from maternal blood into the blastocyst fluid was investigated, during a 30–60-minute interval following parenteral administration, of glucose,

fructose, sucrose; sulphapyridine, thiocyanate. Under the experimental conditions of this study all these substances, except thiocyanate, passed freely into the fluid of the fully implanted blastocyst 8 days after mating. None, however, was able to penetrate into the unimplanted blastocysts. During the initial phase of implantation glucose entered the fluid more readily than either fructose or sucrose.

3. In hyperglycaemic alloxan-diabetic rabbits the glucose concentration in the blastocyst fluid represented only a fraction of that found in the maternal blood.

4. Data are presented for the distribution of glycogen and phosphorus in the rabbit uterus, $7\frac{1}{2}$–8 days after copulation, at the level of implantation, and in the interimplantation areas.

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The Cultivation of the Pituitary of Infantile Rats by the Glass-Rod Technique and the Influence of Grafted Explants on the Growth of Hypophysectomized Hosts

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WITH THREE PLATES

INTRODUCTION

JUST before the last war we made our first attempts to cultivate the anterior pituitary of rats and mice (Martinovitch, 1940). In those preliminary experiments we used pituitaries of animals 1 to 6 months old, and the culture medium was composed of chicken plasma and chicken embryo extract in equal proportions. The explants were grown by the watchglass technique and the incubating temperature was 33° to 34° C. Under these conditions some of our cultures survived for several months. Two months old cultures were grafted in the anterior eye chamber of normal animals and some of these grafts were successfully established. These initial results, and those obtained by Gaillard (1937, 1942), seemed sufficiently promising to justify further research.

Earlier work *in vitro* on the pituitary gland, e.g. the papers of Kasahara (1936), Anderson & Haymaker (1937), and Cutting & Lewis (1938), dealt mainly with unorganized growth. That the pituitary was not cultivated as an organ can be explained by the limitations of the techniques employed. The watchglass method of Fell (1929, 1951) has proved suitable for cultivating fragments or whole organs of embryonic and infantile mammals and birds. Further technical advances were made by Gaillard (1949) when he introduced a dilute medium for the cultivation of endocrine glands, and by Martinovitch, who grew rat and mouse ovaries at subnormal temperatures (1939) and also cultivated pituitary glands by mounting them *in vitro* on the albuginea of the testes of infantile animals (1951). Recently, by introducing a simple modification in Fell's original technique, we have developed a 'dynamic' method of cultivation (1953) by the use of which whole pituitaries of infantile rats have been kept *in vitro* for long periods.

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Using this improved technique, we have (1) studied the nutritional requirements of the explanted pituitaries of infantile rats, (2) followed the histological changes that take place in the organ during prolonged life *in vitro*, (3) investigated the functional potencies of the gland when cultivated *in vitro* and subsequently grafted into hypophysectomized hosts.

MATERIAL AND TECHNIQUE

In these experiments halved anterior pituitaries of 14–17-day-old rats were cultivated in watchglasses (Fell & Robison, 1929) on glass rods (Martinovitch, 1953). The glass-rod technique allows the cultivation of considerably larger pieces of tissues than is otherwise possible. We have taken advantage of this fact to cultivate the whole pituitary of 10–14-day-old rats and the isolated pars posterior of 25–35-day-old animals. Explanted glands were cultivated at 32°–33° C. in rat plasma, chicken plasma, and 25 per cent. chicken embryo extract in the proportions 6 : 2 : 8 or 7 : 2 : 10. In some cultures the following proportions of the nutrient medium were used: 6 drops of rat plasma, 2 drops of chicken plasma, and 4 drops of 50 per cent. chicken embryo extract. For the buffering medium slightly hypotonic Tyrode or Pannett & Compton solutions were used. For the survival of the explanted gland neither of the two salt solutions offer any particular advantage, except that the central region of the explant is rather less degenerate with the Pannett & Compton solution. For the survival of the adrenal gland, on the other hand, I have found that Tyrode solution is distinctly superior. Slightly hypotonic solutions appear to have certain advantages over isotonic ones. In some experiments glucose was added to the medium, but since glucose promotes the migration of cells from the explant, especially during the first 3 weeks, this practice was discontinued. In the presence of glucose also, a marked degeneration in the central region took place, and the restoration of this region required more time than if glucose was not added to the medium. Explants were mounted on glass rods according to the technique described by Martinovitch (1953). The explant was transferred to a new medium once a week.

In order to keep in check the migration of cells from the gland, explants had to be moved once a day to a new position upon the rods.

We found that the glass-rod technique could successfully be used with liquid media. Several rods were placed alongside one another and the explant mounted so as to rest upon two rods. Within 24 to 48 hours the explant fastened itself to the rods which by suitable manipulation could be submerged to any depth desired or kept flush with the surface of the liquid. When the explant is to be transferred to a new medium, the rods with the attached tissue are lifted with a pair of forceps, washed in saline, and placed in a new medium as described above. This technique has been used to study the action of pure serum and other liquid media upon cultivated pituitaries and other endocrine glands. The liquid media used for cultivation both of whole and of isolated anterior pituitaries consisted of rat plasma, chicken plasma, and Pannett & Compton's solution in the

proportion of 12:2:4, or rat plasma and Pannett & Compton's saline, in the proportion of 12:5. For the preservation of granules in acidophil cells these media proved to be superior to those containing embryo extract.

Some anterior pituitaries were mounted on the albuginea of the testis of infantile rats and cultivated as above (see Martinovitch, 1951).

A number of explants kept *in vitro* for 1 to 2 months were grafted in the anterior eye chamber of seven rats hypophysectomized two to eleven months previously and the action of the grafts upon the growth of the host was followed for 3 to 14 months.

Both explanted and transplanted pituitaries were fixed and stained by the Severinghaus technique at various intervals up to 3 months of life *in vitro* and 14 months following grafting.

RESULTS

1. *Appearance of living explants*

When the explants are mounted on glass rods, few cells migrate into the surrounding medium. Migrating cells usually slide along the glass surface and finally lodge themselves along the margin of the zone of liquefaction, where they survive for a few days. Explanted pituitaries retained their healthy, rosy appearance for about 3 weeks. Later, a difference in the transmission of light appeared between the periphery and the centre of the explant, the former becoming translucent and the latter opaque. This difference was probably due to the degeneration of erythrocytes and the degranulation of acidophils near the margin and a massive degeneration in the centre of the explant. It would be misleading to think, however, that opacity of the central region is always the result of degeneration, nor in all explanted glands is translucency a sign of health. For instance, posterior pituitaries explanted alone become very translucent in about 4 weeks, and may then contain nothing but the intercellular substance. Translucency of the anterior pituitaries, however, may be considered a sure sign of their healthy state. A dark amber colour after 3 and more months *in vitro*, again, was a reliable symptom that the process of degeneration was close at hand. Anterior pituitary explants did not liquefy the plasma clot extensively. In cultivation by the glass rod technique, liquefaction of the clot is an advantage as the explant completely loses its contact with the solid medium lying below.

2. *Histological structure of the anterior lobe in explants*

The anterior pituitary whether cultivated in isolation or together with the posterior lobe undergoes various changes. In the peripheral portion, which remains healthy for about 3 months, gradual degranulation of some acidophils and degeneration of others takes place throughout cultivation. Fully granulated or partly degranulated acidophils, however, have been observed after more than 3 months' cultivation (Plate 1, figs. A and B). Usually these cells lie close to the

margin of the explant. One way in which the granules disappear from the acidophil cell is by coalescence (Plate 1, fig. C) to form a red-stained droplet of colloid which may persist after the cell in which it has been formed dies. This coalescence of granules appears to be a symptom of degeneration leading to the destruction of the cell itself. In other cells a gradual degranulation appears to take place, not necessarily accompanied by cellular death. What factors are responsible for the loss of granules is not known.

Early in experimentation it was noted that acidophil granules were best preserved in cultures mounted on the albuginea of the testis particularly in the region protruding from the liquid medium surrounding the explant. At that time we assumed that some simple physical or chemical property of the non-nutrient medium might be responsible for their early disappearance when not protruding from the medium. When, however, a number of explants were cultivated on glass rods in pure liquid media (12 drops of plasma and 5 drops of saline solution), i.e. in the absence of embryo extract, numerous excellently preserved acidophilic cells packed with granules were present in some cultures after 2 months. From these experiments it is clear that chicken embryo extract exercises a deleterious effect upon the granules in some way still unknown. Recently two whole pituitaries were sectioned and stained, one of them after 131 days and the other after 66 days of cultivation. In the first pituitary, cultivated in a medium containing embryo extract, a few acidophil cells are still present (Plate 3, fig. R) and the neurohypophysis appears fairly healthy (Plate 3, fig. S); in the second pituitary, cultivated on glass rods in diluted plasma, both the adeno- and neurohypophysis look remarkably healthy and the explant contains numerous acidophil cells full of granules (Plate 3, fig. T).

In many older explants there are clusters of cells of a type not normally found in the rat pituitary. These cells are small and round with spherical darkly staining nuclei (Plate 1, fig. D); no mitosis has been observed among them. At first we thought that these elements might represent a specialized type of the chromophobe, as apparent transitional forms between the two types were sometimes observed. Now we are more inclined to consider them as acidophils that have lost their granules, and cells with nuclei surrounded by a very thin red-stained ring of colloid can be found among them. In the region occupied by these cells a dark floccular mass of a disintegrating substance fills the intercellular spaces.

The fate of the basophilic cells is still more difficult to follow. As far as we know, none of the staining techniques has proved wholly satisfactory for revealing the basophilic cell in the rat. During the first 5 to 6 weeks of cultivation we are not able to follow the changes that take place within these cells because they are so hard to recognize. After 7 or more weeks of cultivation large blue-stained cells differing from the other two cell types become clearly visible. They have large spherical nuclei, large usually single nucleoli, and abundant cytoplasm stained blue. They occur either individually or in groups of two or three, and always close to the periphery of the explant (Plate 1, figs. E, F, G, and H).

Vacuoles are often present in the cytoplasm of these cells, but we are not certain that they contain granules of any size. In general they very closely resemble the 'castration cells' described by Severinghaus (1939) and others. Closely similar to them and undoubtedly belonging to the same cell type are large clear cells whose cytoplasm may be finely granulated, but fails to take blue colour (Plate 2, fig. I). Only once have we observed a dividing cell that may have belonged to this cell type.

Even after prolonged cultivation the chromophobes do not differ much from the normal cells of the same type and are much more abundant than the cells of the other two types. Mitotic figures are common among them in both young and older cultures, and usually occur near the periphery of the explant (Plate 2, fig. J); degenerating chromophobes are also common. Chromophobes at the periphery of the explant appear somewhat larger than those nearer the centre. During cultivation these cells become divided into nests and chords by narrow strands of connective tissue fibres. Strands of fibres, which appear considerably thicker in whole glands than in explants of the pars anterior, begin at the junction of the adeno-hypophysis and the pars posterior, where they may occupy a fairly large space and send radiating branches which penetrate into the anterior lobe in all directions (Plate 2, fig. K).

Scattered through the cellular mass of the anterior lobe are droplets fairly uniform in size and intensely coloured with fuchsin (Plate 2, fig. L). They are suggestive of degenerating erythrocytes, but this cannot be their source since they are found in cultures that are 2 or more months old. Although there is evidence that the clumped granules of acidophil cells may form droplets which persist for weeks in the explant, it is not certain that all the droplets observed are of one origin. It is possible that the presence of some of the globules, and also of patches of red-stained intercellular substance occasionally found in some explants, may be due to a defective exchange of metabolites between the tissue and the medium; for it was observed that if the medium contained concentrated embryo extract and the saline was isotonic, then large patches of the intercellular substance appeared in the explant; while if, on the other hand, it was more dilute and slightly hypotonic, the patches and the droplets were absent from explants cultivated in diluted rat plasma.

The central region of the explant invariably degenerates (Plate 2, fig. M). The material filling the central necrotic space may have more than one source, but it consists mainly of degenerating acidophils. The only cellular elements that seem to be able to survive degeneration in this region are the chromophobes, which are present throughout the process of degeneration. Owing to this mass degeneration, the explant becomes much smaller, but no central degenerating space is ever formed. The degenerating mass is gradually resorbed and the central region becomes restored through an invasion of white fibres and also by the migration and multiplication of the chromophobes. After the process of degeneration subsides, large bubble-like spaces appear in this region. These

spaces represent deformed vacuoles which blacken with osmium tetroxide and therefore in life contained fat. These fat droplets develop in the cytoplasm of cells which subsequently degenerate leaving the globules behind.

The capsule surrounding the explant remains a thin layer of cells and connective tissue fibres even after prolonged cultivation. This at least partly explains the greater capacity for survival of the pituitary as compared with the adrenal gland where during cultivation the capsule and the subcapsular zone thicken and toughen, thus forming a serious obstacle to the free exchange of metabolites between the medium and the inner portions of the explant (Martinovitch, unpublished).

3. *Histological structure of explants of posterior and intermediate lobes*

We have already noted that the pars posterior, when cultivated separately, has only a limited power of survival, whereas in whole explants it survives as long as the rest of the gland. During cultivation the posterior lobe undergoes changes which make the study of some of its elements rather difficult. In older cultures we invariably encounter one type of cell. These cells have scanty cytoplasm and large nuclei rich in chromatin and intensely stained with fuchsin; they are embedded singly or in groups of two or three in a thick mass of connective tissue (Plate 2, fig. N). Mitotic division has not been observed among them. We have followed the fate of these cells from the first days of cultivation and there is no reason to doubt that they are derived from the original pituicytes.

The cells of the intermedia persist for many weeks. As the explant ages the solid mass of cells is broken up by ingrowing connective tissue fibres into small nests. After 7 or more weeks of cultivation, along the border of the neurohypophysis, small groups of pars intermedia cells enclosed within thick fibrous envelopes are found; they are large and irregular, with spherical nuclei and dark purple cytoplasm (Plate 3, fig. O).

In whole pituitaries soon after explantation a deep cleft begins to appear between the intermedia and adeno-hypophysis, the old hypophysial cavity (Plate 3, fig. P). With the ageing of the explant a cavity is formed, which may become greatly enlarged, filled with a fluid in which fragments of degenerating cells are scattered; this cavity is lined with epithelial cells one or two layers thick. In older cultures the original cavity may break up into a number of smaller spaces surrounded by columnar cells several layers thick. In the walls lining the cavity dividing cells were found in some 2 months old cultures.

The fate of the pars tuberalis has not been followed in these experiments owing to the insignificant mass of tissue forming this portion of the gland in the rat.

4. *Histological structure of pituitary explant-transplants*

The histological picture of the pituitary explants following transplantation to the anterior chamber of the eye of host rats does not change much. Explants

cultivated *in vitro* for 5 and 6 weeks when grafted occasionally show some poorly granulated acidophils distributed along the periphery of the transplant, the rest of the cells being of the non-granular type. Such acidophils are more likely to appear in transplanted cultures that have been kept *in vitro* 3 and 4 weeks than in those that have been cultivated for longer periods. Transplanted explants grown 8 and 9 weeks *in vitro* almost uniformly contain one type of cell, the non-granular.

Direct transplants of the anterior pituitary without previous cultivation *in vitro*, after prolonged life in the anterior eye chamber of a host animal, present essentially the same histological picture as that observed in the pituitary explant-transplants under the same conditions of survival.

After grafting the blue cells observed in explanted glands do not appear. In the whole pituitary explants following grafting the pituicytes persist; very likely cells of the pars intermedia are also there, but they cannot be recognized as such. Remnants of the old hypophysial cavity represented by a number of chord-like formations with and without lumina are still present in the transplant. In general the transplanted explant presents a simplified histological picture and does not return to the normal state (Plate 3, fig. Q).

5. *The growth response of hypophysectomized rats to explant-grafts*

It is known that in hypophysectomized rats growth is completely arrested. When young rats, hypophysectomized at the age of 60 to 90 days, receive 2 to 11 months after the operation grafts of four to six pituitaries of infantile rats (10 to 17 days old) cultivated *in vitro* for 1 to 2 months, the animals immediately

TEXT-FIG. 1

resume their arrested growth. Growth proceeds at a much slower rate than in the normal animal and it may continue for several months. During this post-transplantation period the weight of the host animal may rise considerably, but the average maximal size, typical for the strain, is never reached. For an illustration we present the growth curve (Text-fig. 1) of a rat which 11 months following hypophysectomy received five anterior pituitaries cultivated *in vitro* 32 days. After grafting this animal resumed its growth and continued to gain in weight for many months, more than doubling its original weight during that time. Six other hypophysectomized rats with explant grafts reacted in the same way (see Martinovitch, 1950). In some of the grafted explants the Severinghaus technique revealed numerous acidophilic cells, in one case after 13 months of life in the anterior eye chamber of the host animal; in other cases only one type of cell was found, the non-granular.

Pituitaries kept *in vitro* for shorter periods give better grafts, as shown by the fact that following grafting, in a number of cases, a visible increase in the mass of tissue took place. Animals first grafted then hypophysectomized respond more readily, as demonstrated by their gain in weight, to the pituitary explant-transplants, as they also do to direct transplants of the pituitary gland (Martinovitch, 1949; Martinovitch & Vidović, 1953).

DISCUSSION

In the experiments described above we have shown that halved anterior pituitaries of 14–17-day-old rats and whole pituitaries of 10–14-day-old animals can be kept *in vitro* in watchglasses on glass rods for at least 3 months. The glass-rod technique has proved especially satisfactory for the whole pituitary explants. It is an interesting fact that after an almost complete primary degeneration of the central tissue of the gland this region eventually becomes restored, i.e. filled with cells of purely non-granular type and connective-tissue elements. These readjustments, as would be expected, are accompanied by a considerable reduction in the size of the original explant. Another interesting observation was that the posterior lobe, or at least some elements comprising this portion of the gland, survives as long as the anterior lobe. In an earlier publication (1953) we have shown that isolated posterior lobes of 25–35-day-old rats can survive *in vitro* for 3 to 4 weeks, whereas posterior pituitaries of younger animals cultivated in the same way disintegrate in a few days. It is our opinion that, in the latter case, the minute size of the explant and the relatively large surface exposed to the action of the surrounding medium is chiefly responsible for this reaction, for usually younger tissues and organs adapt themselves to conditions *in vitro* better than older material. Posterior lobes in whole explants have a much smaller area exposed to the direct action of the medium, hence their longer survival.

Both Kasahara (1936) and Gaillard (1942, 1948) have noted a gradual degranulation of migrating chromophilic cells of the anterior pituitary of the rabbit *in vitro*, and the latter author observed the same phenomena in the mother

explant itself. The granules also disappear from some of the acidophilic cells in rat pituitaries, but in many explants acidophils full of intensely stained granules have been noted after 4 and 5 weeks, and in one instance after 3 months of cultivation. Partly degranulated acidophils can be found in the explanted pituitary throughout the culture period. No clear-cut evidence has been obtained to show whether the degranulated acidophils are able to regenerate their granules either *in vitro* or after being grafted into an animal. The factors responsible for the degranulation of the acidophils are obscure. All that we know is that cells close to the periphery of the gland retain their granules longest both *in vitro* and in the transplants, and that in media containing embryo extract the granules tend to coalesce and form a droplet of colloid which may persist long after the cell giving rise to them has died.

Changes that lead to a reduced complexity of this original histological structure are superseded by new features some of which, undoubtedly, are degenerative phenomena. Other changes, however, cannot be classed as such, e.g. the appearance of large blue cells suggestive of the castration cell type is a feature not previously found in pituitaries of infantile rats. Clusters of small cells closely packed together and rich in chromatin probably represent another new feature. We have not been able to find them in the normal pituitary gland and, as far as we know, they have not hitherto been described. We believe that they represent acidophils that have lost their granules, and that their appearance depends upon the submergence of the explant in highly diluted media containing embryo extract.

By making confronted cultures of the anterior pituitary of the rabbit and osteogenic fibroblasts, Gaillard (1948) observed a stimulating effect of the pituitary upon the neighbouring culture; to quote the author: 'After a longer cultivation (20–30 days), the histological picture of the explant appeared to be changed. The granular elements disappeared and only a nongranular type of cells remained. Nevertheless these explants were able to produce a growth-promoting substance (growth hormone) as tested on confronted pure strain cultures of the fibroblast type.' On the other hand, discoveries that gigantism in men is accompanied by acidophilic adenomas and that acidophils are absent from pituitary of dwarf rats have led endocrinologists to think that these cells are producers of the growth-hormone.

Explant-transplants may offer a more promising approach to the study of the hormone-producing capacities of the non-granular cells. Although in some of the grafted explants the Severinghaus technique revealed numerous acidophilic cells full of granules, others contained only one type of cell, that is the chromophobe. Yet such transplants lodged in the anterior chamber of the eye caused hypophysectomized hosts to resume growth that was arrested at hypophysectomy. It would make an interesting problem to explant for a second time an explant-transplant of this type together with a receptor tissue. Ample evidence, however, has been gathered in these experiments to show that explanted glands,

even after prolonged cultivation, do not lose their capacity for manufacturing growth hormone.

Finally, something should be said about the advantages that the glass-rod technique offers for the survival of explanted endocrine glands. Like all endocrines the anterior pituitary is sensitive to the culture medium, but less so than the adrenal gland. In cultures mounted on a plasma clot the constant emigration of cells disrupts the original morphology of the explant and makes it more difficult to study the effects of the culture medium upon the gland. A gradual sinking of the explant into the medium, owing to the action of tissue enzymes upon the underlying clot, aggravates the situation still more. The reaction of the exposed parenchyma cells to the culture medium is quite different from what it is when these cells are protected by a capsule of connective-tissue cells and fibres. No parenchyma cell survives longer than a couple of weeks at most if directly exposed to the action of the medium; protected by the capsule it may live for months (see Gaillard, 1949). Cultivating pituitary glands on glass rods almost eliminates cellular outgrowth and prevents the tissue from sinking into the medium; it also allows a better appreciation of what a particular medium can do for the survival of the explant as a whole. Moreover, it is probable that the glass-rod technique permits an intimate contact between the tissue and the gaseous medium of the atmosphere and also offers a rich supply of the liquid medium, which can be produced by pushing the explants to a new place on the rods. Both of these are advantages of the roller tube cultures of Dr. Gey (see Feller, 1950), a method that is similar to our glass-rod technique in this respect. The glass-rod technique is also advantageous for experiments in which liquid media are preferable to a solid clot.

SUMMARY

1. Explanted anterior pituitaries of 13–17-day-old rats survive in watchglasses on glass rods for at least 3 months.

2. In whole explants of 10–14-day-old animals the posterior lobe (pars nervosa), and probably the pars intermedia as well, survive as long as the anterior lobe. Both the anterior lobe and the pars nervosa survived, when embryo extract was added to the medium, for up to 131 days.

3. Some acidophilic cells, partly degranulated, persist in cultures throughout the whole *in vitro* life of the explant.

4. Basophils closely resembling the 'castration cell' are clearly visible in stained preparations of the explant, but only after 6 to 8 weeks of cultivation.

5. A number of dividing chromophobes have been observed during the first 2 months of cultivations.

6. Anterior pituitaries after cultivation for between 1 and 2 months when grafted into the anterior eye chamber caused young hypophysectomized rats to resume their growth which had been completely arrested.

7. In some explant-transplants the parenchyma of the anterior lobe consists

of only one type of cell, namely the chromophobe; in others, some partly granulated acidophils have been found. Basophils have not been observed in transplanted explants.

8. Anterior pituitaries cultivated in a diluted (liquid) plasma medium retain a remarkably normal appearance for over two months and contain numerous healthy-looking acidophil cells throughout that time.

9. A modification of the glass-rod technique for use with liquid media has been described.

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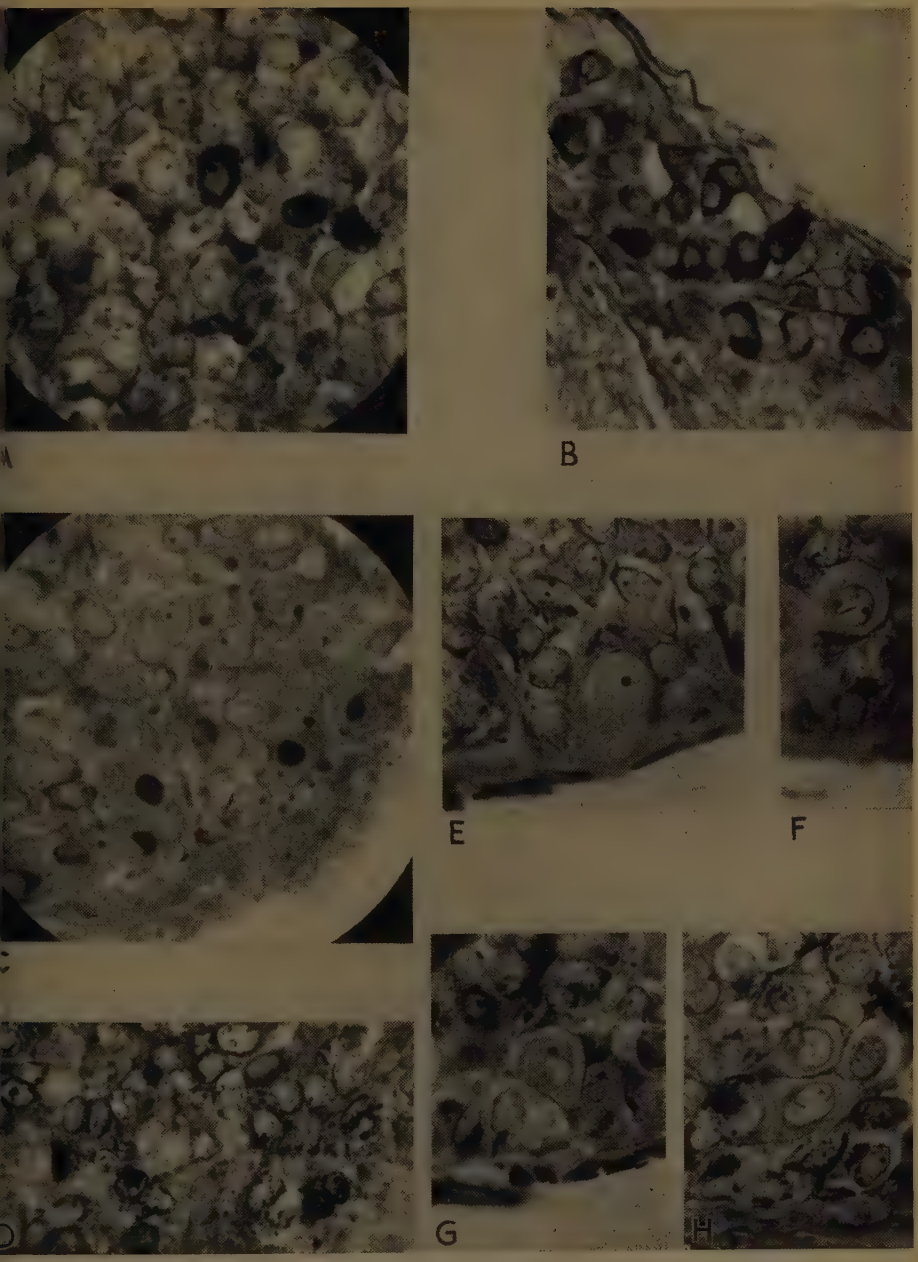

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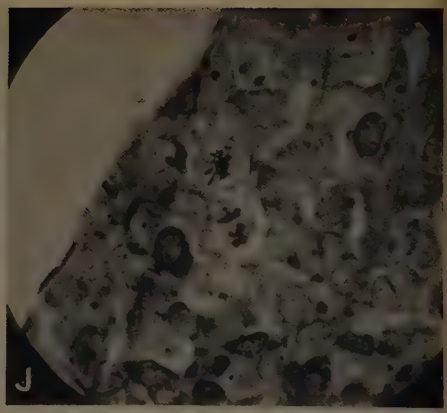
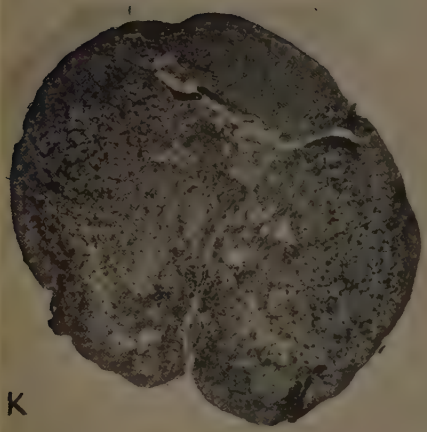
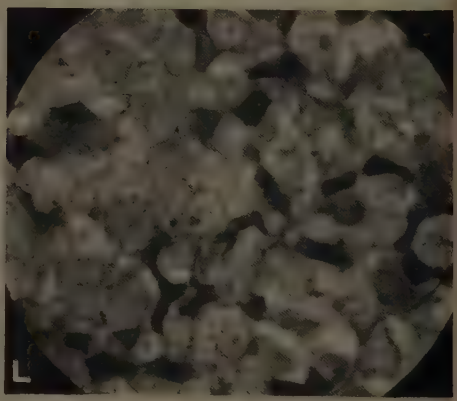
Plate 1







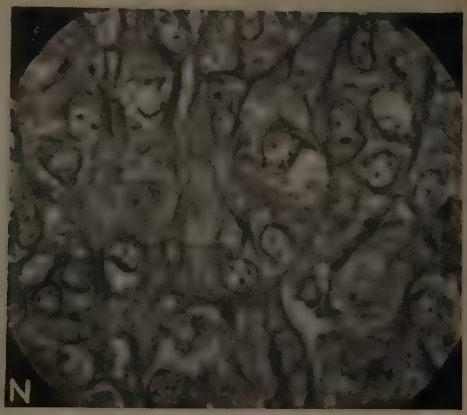


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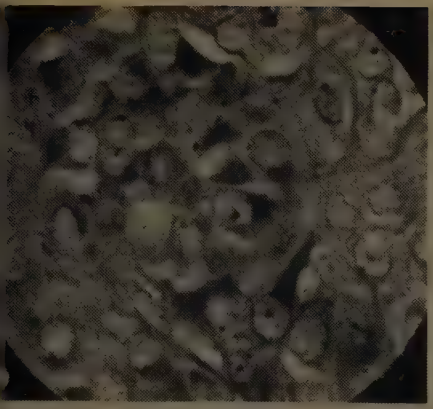
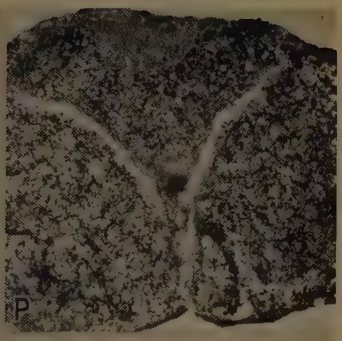
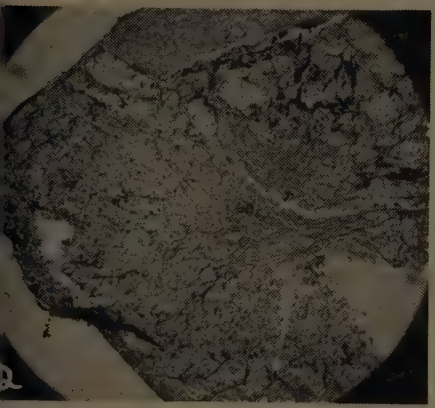
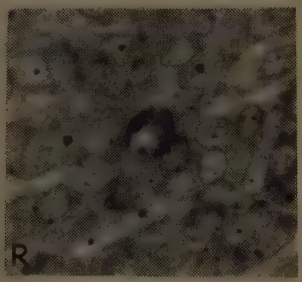
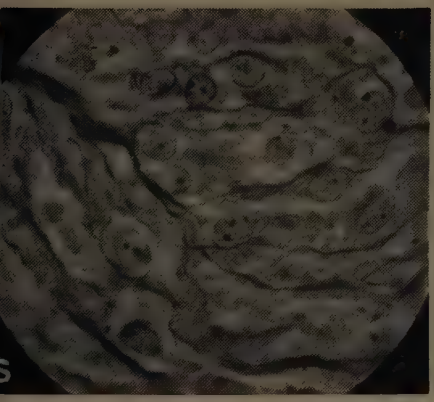
Plate 2

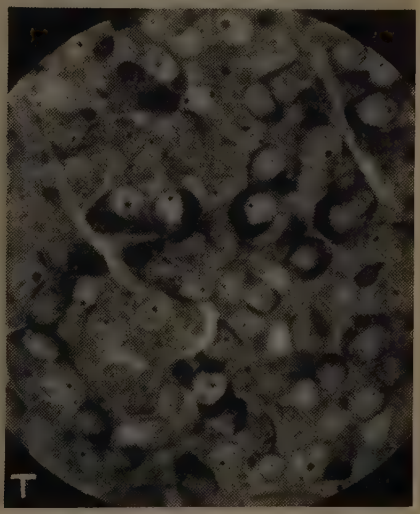












P. N. MARTINOVITCH

EXPLANATION OF PLATES

All the explanted pituitaries have been stained by the Severinghaus technique. Figs. K, M, P, and Q are magnified 90, figs. B and R 730, and all other figures 920 diameters.

Plate 1

Fig. A. Three acidophil cells surrounded by numerous chromophobes in the isolated anterior pituitary of a 14-day-old rat after 58 days *in vitro*. In the cell at the extreme right granules have coalesced to form a droplet.

Fig. B. Acidophil cells full of granules in a 91-day-old culture of the anterior pituitary of a 13-day-old rat. This pituitary explant was mounted on the albuginea of the testis of an infantile animal.

Fig. C. Anterior pituitary of a 14-day-old rat after 58 days *in vitro*. Note coalescing granules within the cytoplasm of the acidophil cells, forming large droplets of colloid.

Fig. D. A cluster of degranulated acidophils from the anterior pituitary of a 14-day-old rat after 58 days *in vitro*. (For comparison see fig. M.)

Figs. E, F, G, and H. Large blue-stained cells from the anterior pituitary of a 14-day-old rat after 58 days *in vitro*.

Plate 2

Fig. I. A large, clear cell from the anterior pituitary of a 14-day-old rat after 58 days *in vitro*.

Fig. J. Two dividing chromophobes from the anterior pituitary gland of a 14-day-old rat after 58 days *in vitro*.

Fig. K. Section through a whole pituitary gland of a 14-day-old rat after 58 days of cultivation. Above, the neurohypophysis flanked with tiny patches of intermedia tissue. Below, two halves of the anterior pituitary.

Fig. L. Same explant as fig. K. Scattered through the mass of tissue of the anterior pituitary gland are 'droplets' of red-stained colloid.

Fig. M. Section through the anterior pituitary of a 13-day-old rat, 25 days *in vitro*. Note darkly stained centre in process of degeneration.

Fig. N. Section through the neurohypophysis of a 13-day-old rat, 58 days *in vitro*.

Plate 3

Fig. O. Pars intermedia cells from a whole pituitary gland of a 13-day-old rat, 58 days *in vitro*, forming a narrow strip of tissue between the nervosa (left) and pars anterior.

Fig. P. Section through a whole pituitary gland of a 13-day-old rat after 25 days' cultivation. The cleft separating intermedia from adenohypophysis is well shown. Note: V-shaped intermedia above, two halves of pars anterior below, and a semicircular area of neurohypophysis above the intermedia.

Fig. Q. Section through a whole pituitary explant-transplant of a 14-day-old rat, 58 days *in vitro* and 37 days in the anterior eye chamber of a hypophysectomized host. Pars nervosa above.

Fig. R. A well-preserved acidophil cell in the anterior pituitary of a 13-day-old rat after 131 days of cultivation in rat plasma, chicken plasma, and chicken embryo extract.

Fig. S. Section through the neurohypophysis of the same explant as in fig. R, showing a few pituicytes in a healthy state.

Fig. T. Numerous acidophil cells in the pituitary gland of a 10-day-old rat, after 66 days of cultivation in diluted (liquid) rat plasma. Note the healthy appearance of the tissue of this explant.

Observations on the Nature of the Relationship between Cell Division and Carbohydrate Metabolism

by R. J. O'CONNOR[1]

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INTRODUCTION

ALTHOUGH there is considerable evidence that carbohydrate metabolism plays an important part in the process of cell division (see Bullough, 1952), conclusions have differed regarding the relative importance of glycolysis and catabolism involving respiration. In the adult mouse epidermis Bullough & Johnson (1951) found that cell division was dependent upon the respiratory oxidation of pyruvate by the tricarboxylic acid cycle. On the other hand, Pomerat & Willmer (1939) showed that, in tissue culture, agents that inhibited respiration had little immediate effect on cell division, while Laser (1933) found that growth of cultured fibroblasts could continue when respiration was greatly diminished by low oxygen tensions. Further, Warburg (1930) concluded that cell growth is associated with glycolysis.

A similar difference in the relationship of mitotic activity to the respiratory and glycolytic forms of carbohydrate metabolism was indicated by changes occurring in the midbrain and the red-blood cells of the chicken embryo during embryonic development. In the midbrain (O'Connor, 1950*a*) there was a corresponding decrease in the number of dividing cells in a unit volume of tissue and in the rate of aerobic glycolysis as measured by the production of acid by midbrain tissue isolated in a glucose containing medium (acid formation did not occur when glucose was omitted from the medium; O'Connor, 1949). Under these conditions the rate of respiration remained constant. Since the respiratory quotient was unity, this constant rate of respiration was taken to indicate a constant rate of glucose utilization by respiratory metabolism. Further investigations (O'Connor, 1950*b*) showed that, when the rate of aerobic glycolysis of the isolated midbrain was decreased by fluoride or iodoacetate, cell division was inhibited by concentrations which did not diminish the rate of respiration nor alter the respiratory quotient. Thus it was concluded that, in the midbrain, cell division was dependent on aerobic glycolysis, as measured by the acid production of the isolated tissue in a glucose-containing medium.

When the red-blood cells were investigated in a similar medium (O'Connor,

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1951) it was found that acid formation did not occur but the rate of respiration decreased during development in a manner corresponding to the decrease in the proportion of dividing cells. The respiratory quotient of the red-blood cells was unity, so that these findings suggested that there was a corresponding decrease in mitotic activity and the catabolism of glucose by processes involving respiration. This suggestion was supported by subsequent investigations which showed that the inhibition of respiration by fluoride and by iodoacetate resulted in inhibition of cell division (O'Connor, 1952). Mitotic activity in the red-blood cells and in the midbrain thus differs in its relationship to carbohydrate catabolism, since in the former it is associated with the respiratory utilization of glucose, and in the latter with aerobic glycolysis, as measured by the acid production of the isolated tissue in a glucose-containing medium.

In order better to assess the significance of this difference, comparisons have been made of the changes undergone by mitotic activity and carbohydrate metabolism in the developing liver of the chicken embryo, the metabolism of which differs in certain relevant respects from that of the midbrain and red-blood cells. As shown previously (O'Connor, 1953*a*), in isolated liver tissue, the respiratory quotient is less than unity, indicating that oxygen is utilized in the metabolism of substrates other than carbohydrate. Further, the carbohydrate metabolism of the liver is complicated by the deposition of considerable amounts of glycogen during the course of its development.

MATERIAL AND METHODS

All observations were made on the liver of chicken embryos incubated at 38° C. The eggs were taken from the batches used previously (O'Connor, 1953*a*), and the observations were scattered at random among the batches. As previously, the embryos were grouped in arbitrarily chosen stages of development. Each stage represents an increase in the eye diameter of 0·7 mm. and is referred to below by its median eye diameter (M.E.D.). The mean time of incubation for each of these stages has already been recorded (O'Connor, 1953*a*), and, since the eggs used in these experiments are from the same batches, these times are applicable to the present observations and are repeated in Table 1.

The estimation of glucose utilization by isolated liver tissue

In the accepted scheme of carbohydrate catabolism in animal tissues the substrate, whether glucose or glycogen, is first transformed into pyruvic acid. In the presence of oxygen, part or all of the pyruvic acid is oxidized by way of the tricarboxylic acid cycle with the consumption of oxygen. On the other hand, even in the presence of oxygen, part of the pyruvic acid may be reduced to lactic acid. These two processes, respiratory catabolism and aerobic glycolysis, have been separately estimated from observations on the gaseous exchange of isolated liver tissue.

(a) Acid production by isolated liver tissue. In order to determine the rate of

aerobic glycolysis of the isolated liver, the rate of total acid production was measured by a Cartesian diver micromanometer. Although the technique used has already been recorded (O'Connor, 1950*a*), it possesses certain limitations requiring present consideration and is, therefore, recapitulated in part.

Livers were removed from the embryos and placed in the following medium: NaCl 0·9 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., glucose 0·20 g., water 100 ml., to which was added 10 ml. M/15 phosphate buffer (Sørensen) to produce pH 7·4; this is the medium previously used (O'Connor, 1953*a*) to determine the rate of respiration and the respiratory quotient. As in those determinations, fragments of liver were cut from the anterior surface of the right lobe of the liver and the volume measured; this volume varied from 0·3 to 0·8 c.mm. The fragments were then washed twice in the following medium: NaCl 0·9 g., KCl 0·02 g., $MgCl_2$ 0·02 g., $CaCl_2$ 0·02 g., glucose 0·20 g., $NaHCO_3$ 0·20 g., water 100 c.c. The liver fragments were then introduced into divers containing this medium. The gaseous phase of the divers was 95 per cent. O_2: 5 per cent. CO_2 and the arrangement of fluids within the divers, as well as their dimensions, were as described previously (O'Connor, 1950*a*). In such divers it should be noted that there is no alkali to absorb carbon dioxide so that the manometric reading is affected not only by carbon dioxide released from the bicarbonate in the medium as the result of acid formation, but also by the respiration of the liver tissue and by the carbon dioxide formed as a consequence. In order to take account of the last two factors the assumption was made that they occurred at the same rate under the conditions of the present observations as they did under the conditions in which respiration and respiratory quotient were investigated previously (O'Connor, 1953*a*), when the medium contained phosphate and not bicarbonate (see above) and the gaseous phase of the divers was oxygen. Using these results, a figure was calculated which represented the amount of carbon dioxide presumed to result from acid formation, and this will be referred to as 'presumed acid formation'. In arriving at this figure the factor was calculated, both for oxygen and carbon dioxide, which related manometric change to the change in gaseous content of the diver. The formula given by Boell, Needham, & Rogers (1939) was used. Comparable observations and calculations were made when the isolated liver was suspended in the bicarbonate-containing medium from which glucose was omitted. All observations were made over a period of 2 hours, during which time the rate of change in the manometric reading did not vary significantly in any one particular observation.

(b) Respiratory utilization of glucose by isolated liver tissue. Since, as mentioned above, the oxygen consumed by isolated liver tissue is used in the catabolism of substrates other than carbohydrate, the rate of respiration cannot be used directly as a measure of the respiratory catabolism of glucose as it was in the case of the midbrain and red-blood cells. It was considered, however, that this rate could be estimated from the decrease in the rate of respiration which resulted when glucose was omitted from the medium in which respiration was

measured. Rates of respiration of the isolated liver in the presence and absence of glucose have been recorded previously (O'Connor, 1953*a*), and these were used in the present investigations to calculate the rate of the respiratory utilization of glucose at different stages of development.

The estimation of mitotic activity of the liver cells

This was carried out by determining, from histological sections, the percentage of hepatic cells in mitosis. Cells other than hepatic cells were not considered. Sections cut at a thickness of 6μ from livers fixed in Bouin's fluid were stained with iron haematoxylin. No counter-stain was used.

RESULTS

Glucose catabolism of isolated liver tissue

(a) Rate of 'presumed acid formation'. The mean value and the standard error of the mean is recorded for each developmental stage in Table 1, and results obtained in the presence and absence of glucose are given. In the latter case the values obtained were so regularly in the vicinity of zero that the results for more than one stage of development were treated together when the mean values and standard errors were determined.

TABLE 1

Metabolism and cell division in the liver of the chicken embryo

Stage of development (M.E.D. mm.)	*Mean time of incubation (days)*	*'Presumed acid formation' (μl. CO_2/c.mm. liver/hr.)*						*Mitotic activity (dividing hepatic cells, per cent.)*			*Respiratory utilization of glucose, μl. O_2/c.mm. liver/hr.*	
		With glucose			*Without glucose*							
		N.	*M.*	*S.E.*	*N.*	*M.*	*S.E.*	*N.*	*M.*	*S.E.*	*M.*	*S.E.*
2·5	5·5	11	0·89	0·11	8	−0·01	0·06	8	1·43	0·14	0·37	0·08
3·2	6·1	12	0·81	0·14				4	1·38	0·07	0·48	0·10
3·9	6·5	9	0·28	0·08	6	−0·03	0·04	7	1·10	0·11	0·26	0·13
4·6	6·9	6	0·18	0·10				9	0·81	0·03	0·07	0·04
5·3	7·3	5	0·04	0·11	6	0·08	0·10	6	0·64	0·07	0·04	0·08
6·0	7·8	8	0·05	0·08				4	0·64	0·06	0·06	0·01
6·7	8·4	5	0·06	0·09	5	0·07	0·06	4	0·62	0·11	0·00 (*a*)	0·07
	10	6	0·03	0·08								

M.E.D., median eye diameter; N., number of observations; M., mean; S.E., standard error; (*a*), the value actually obtained was −0·08, which in view of the standard error can be considered as nil. The metabolic measurements were made in conditions of isolation described in the text.

(b) Rate of respiratory catabolism. The differences in the mean values for the rate of respiration in the presence of glucose and its absence, by which respiratory utilization of glucose is measured, are recorded for each of the developmental stages in Table 1. The standard error given for each difference is the standard error of the difference of the two means concerned and has been calculated from data already recorded (O'Connor, 1953*a*).

Mitotic activity in liver cells

To determine the percentage of hepatic cells in mitosis 2,000–3,000 cells were examined in each individual case. The mean value for a number of embryos in each developmental stage and the standard error of the mean are recorded in Table 1. Since the nuclei of dividing and resting cells differ in size an error is presumably introduced (Abercrombie, 1946). This error, however, would not differ greatly at different stages of development and would not, therefore, diminish the significance of the decrease during development of the percentage of cells seen in mitosis and particularly between the stages of M.E.D. 3·2 mm. and M.E.D. 4·6 mm.

DISCUSSION

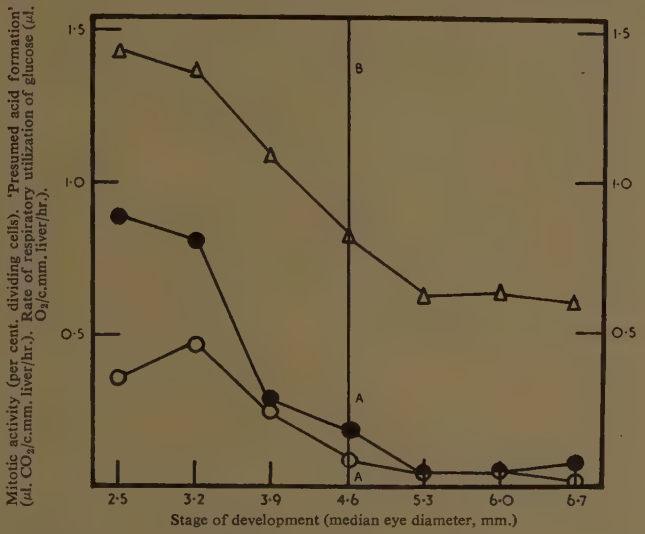
Aerobic glycolysis by isolated liver tissue

The rates of 'presumed acid formation' were calculated in order to measure rates of aerobic glycolysis, that is the production of lactic acid from glucose. However, before the results are used for this purpose certain considerations are necessary. It will be recalled that the 'presumed acid production' was calculated on the assumption that the rate of respiration and the respiratory quotient were the same under the conditions of the present observations as in the conditions under which they were previously determined (O'Connor, 1953*a*); Dixon (1951) has pointed out that this assumption may not be justifiable. However, the gaseous exchange concerned, namely the uptake of oxygen and the resulting evolution of carbon dioxide, would affect manometric readings in opposite directions and so decrease any error introduced. Also, none of the results recorded in Table 1 for 'presumed acid formation' are significantly below zero. Since negative results can be considered 'absurd', their absence is in favour of the calculation being based on correct assumptions. Therefore, even if the possibility of error is not completely eliminated, it is considered that 'presumed acid formation' sufficiently represents true acid formation by isolated liver tissue to conclude that during development this decreases to reach a rate of nearly zero at stages of M.E.D. 5·3 mm. and later (see Table 1). In a previous publication (O'Connor, 1953*a*) the evidence was considered which excludes the possibility that this decrease is due to the addition, during development, of metabolically inert substances to the hepatic cells.

Although uric acid may be formed by isolated liver tissue (O'Connor, 1953*a*), the amount of carbon dioxide it releases from bicarbonate is unimportant because when glucose is omitted from the medium, acid formation nearly disappears (Table 1). Thus the acid formed is nearly, if not completely, derived from glucose, and can therefore be presumed to be lactic acid. For these reasons it is considered that 'presumed acid formation' is sufficiently a measure of lactic acid production from glucose to permit its use as a measure of aerobic glycolysis.

The relationship of cell division to carbohydrate metabolism

Using 'presumed acid formation' as a measure of aerobic glycolysis the rate of this process at different developmental stages has been compared, in Text-fig. 1, with corresponding rates of respiratory glucose utilization and with mitotic activity. Line *AB* is added to the figure to indicate the first appearance of glycogen in embryos of the stage of M.E.D. 4·6 mm. These embryos have a



TEXT-FIG. 1. Mitotic activity (Δ————Δ), rate of 'presumed acid formation' (●————●), and the rate of the respiratory utilization of glucose (○————○) of the liver tissue at different stages of development. The metabolic measurements were made as described in the text, where reasons are given for regarding 'presumed acid formation' as a measure of aerobic glycolysis. Line *AB* indicates the first appearance of glycogen in the liver cells.

mean incubation time of 6·9 days (O'Connor, 1953*a*). The figure shows that with the appearance of glycogen there is an alteration in the relationship between mitotic activity and the glucose metabolism of isolated liver tissue. Before glycogen appears there is a corresponding decrease in the proportion of dividing cells, the rate of 'presumed acid formation', and the rate of the respiratory utilization of glucose (Text-fig. 1). After the appearance of glycogen these metabolic processes almost disappear, although dividing cells persist and constitute about 0·6 per cent. of hepatic cells. Furthermore, both before and after the appearance of glycogen the relationship between mitotic activity and glucose metabolism by the isolated tissue differs from that found in the midbrain and in the circulating red-blood cells, for in the former decreasing mitotic activity is associated with decreasing acid production from glucose (O'Connor, 1950*a*), while

in the latter the association is with decreasing respiratory metabolism (O'Connor, 1951). These differences are summarized in Table 2, and it is possible that they represent a significant difference in the metabolic relationships of mitotic activity in the four tissues. On the other hand, the similarity of the mitotic mechanism in these and other tissues suggests the alternative possibility that

TABLE 2

Changes in the rate of carbohydrate metabolism associated with the decrease in mitotic activity which occurs during the development of tissues in the chicken embryo

	Rate of carbohydrate metabolism	
Tissue	*Respiratory*	*Producing acid*
Midbrain (O'Connor, 1950*a*)	Remains constant	Decreases as mitotic activity decreases
Red-blood cells (O'Connor, 1951)	Decreases as mitotic activity decreases	Absent
Liver before glycogen appears (see text)	Decreases as mitotic activity decreases	Decreases as mitotic activity decreases
Liver after glycogen appears (see text)	Very low	Very low

The rates of carbohydrate metabolism are those of the isolated tissue in a glucose-containing medium. In the references quoted and in the text reasons are given for regarding acid production as a measure of aerobic glycolysis.

the differences recorded in Table 2 are nevertheless an expression of a common dependence of cell division on carbohydrate catabolism, as has been suggested by Bullough (1952) and which, in the case of the midbrain and the red-blood cells, has been indicated by the correspondence between the effect on cell division and the inhibition of carbohydrate catabolism produced by fluoride and iodoacetate (O'Connor, 1950*b*, 1952).

In the case of the midbrain, red-blood cells, and the liver before the appearance of glycogen, the findings of Table 2 could be accounted for if mitotic activity were dependent on the total amount of pyruvate disposed of by conversion to lactic acid in aerobic glycolysis together with its oxidation by the tricarboxylic acid cycle. Thus in the case of these three tissues it is possible that the reactions by which pyruvate is formed from glucose play an essential part in cell division. Such reactions could occur in the liver after the appearance of glycogen, in spite of the findings in Table 2, if pyruvate or compounds intermediate in its formation were incorporated into larger molecules. Such reactions leading to glycogen formation are well known in the adult liver, and it is likely that they occur in the embryonic liver after the appearance of glycogen, for Dalton (1937) has concluded that at this stage the liver of the chicken embryo becomes capable of adult functions. Thus the findings in all four tissues recorded in Table 2 could be accounted for on the assumption that mitotic activity was

dependent on the reactions leading to formation of pyruvate from glucose. Although there is not sufficient evidence to establish such a relationship, it is possible to consider its implications and compare them with results obtained by other investigators. Such is the purpose of the following discussion.

There is considerable evidence that in the metabolism of glucose there is a pathway alternative to the glycolytic pathway of the Meyerhof scheme. In this hexose monophosphate oxidative route[1] glucose is converted into ribose-5-phosphate by reactions which involve the consumption of oxygen (Dickens & Glock, 1951). In the further metabolism of ribose-5-phosphate, a triose is formed which is incorporated into glucose-6-phosphate. Evidence for such reactions has been found in a number of tissues which include, in the adult, the liver (Glock, 1952), the red-blood cells (Dische, 1951), and brain tissue (Sable, 1952). Pyruvate could be formed as a result of such reactions since both the triose and the glucose-6-phosphate could be acted upon by enzymes of the glycolytic system. Thus the possibility that mitotic activity depends upon reactions leading to pyruvate formation gives rise to the further possibility that the actual reactions concerned may be those associated with the formation of ribose-5-phosphate. Since ribose-5-phosphate is similar to the pentose component of ribonucleic acid, this possibility would be in accordance with the well-established association of cell proliferation and ribonucleic acid (Caspersson, 1950; Brachet, 1947). Further, the association of cell division with the alternative hexose monophosphate oxidative route of glucose metabolism might constitute a basis for an explanation for a special form of metabolism which, it has been claimed, is associated with embryonic development (cf. Moog, 1944).

However, pyruvate formation by the hexose monophosphate oxidative route with ribose-5-phosphate as an intermediate compound involves the consumption of oxygen. Therefore, to suggest that such a process plays a dominant part in cell division makes it necessary to consider objections that might arise from observations suggesting that cell division can occur independently of oxygen consumption. In the case of the observations made on the isolated midbrain tissue, and recorded in Table 2, the association of decreasing mitotic activity with a constant rate of oxygen consumption might be the basis of such an objection. However, modifications of enzyme activity occurring in the course of differentiation might be adequate to meet this objection, since it is possible that any decrease of the oxygen utilized in the formation of ribose-5-phosphate may be balanced by an increase in the proportion of pyruvate oxidized by the tricarboxylic acid cycle. After the appearance of glycogen in the liver of the chicken embryo it might be questioned whether the oxidative formation of ribose-5-phosphate could occur because of the low values obtained for oxygen consumption associated with the catabolism of glucose (see Table 1). However,

[1] There has been discussion about the most appropriate name for this metabolic pathway (Dickens, 1953). The term 'hexose monophosphate oxidative route', suggested by Dickens, will be used.

since measurements were made on isolated liver tissue they would not exclude the presence of the hexose monophosphate oxidative route in the intact embryonic liver, particularly since, in the adult liver, the necessary enzymes have been demonstrated (Dickens & Glock, 1951), and it is to be expected that such enzymes would be present in the liver of the chicken embryo after glycogen appears because of the evidence that, at this period of development, the embryonic liver is capable of adult function (Dalton, 1937).

Apart from the results recorded in Table 2 the possibility of a dependence of mitotic activity on an oxygen-consuming process might appear inconsistent with observations that, in some cells, division can be completed in the absence of oxygen (e.g. Lettré, 1951). Although, in such circumstances, glycolytic reactions could continue and be adequate to meet the energy requirements of the cell, the reactions leading to ribose-5-phosphate formation would presumably cease. However, if cell division were dependent on ribose-5-phosphate formation, its failure in such conditions might not inhibit mitosis, at least for a time, since the failure of ribose-5-phosphate formation might be balanced by a decreased rate of destruction. Further, if ribose-5-phosphate is concerned in the synthesis of ribonucleic acid (Cohen, 1951) any failure of its formation might be met by utilization of precursors of nucleic acid in the cell (cf. Walker & Yates, 1952).

Even if it were established that cell division was dependent on the reactions leading to pyruvate formation, it is unlikely in ordinary circumstances that pyruvate formation, whether by the glycolytic or by the hexose monophosphate oxidative route, meets the energy requirements of the dividing cell because the greater part of the energy produced in carbohydrate catabolism comes from the oxidation of pyruvate by the tricarboxylic cycle (Burton & Krebs, 1953). Further, Bullough & Johnson (1951) have shown by experiment that, in the isolated adult mouse epidermis, cell division is dependent on the oxidation of pyruvate in the tricarboxylic acid cycle. In most cells this would constitute a dependence of mitotic activity on carbohydrate catabolism, since pyruvate is derived from carbohydrate and in cells with a respiratory quotient of unity carbohydrate may be its sole source (e.g. the midbrain tissue and red-blood cells of the chicken embryo; O'Connor, 1950*a*, 1951). However, in the case of the liver after the appearance of glycogen it may be necessary to make an exception, because the respiratory quotient of isolated liver tissue is 0·69, which suggests that the oxygen consumed is utilized in the formation of uric acid from protein (O'Connor, 1953*a*). In this case, therefore, it may be that the energy requirements of dividing cells are met by the catabolism of protein rather than of carbohydrate. It is relevant to note that after glycogen appears no difference could be detected, by histochemical methods, in the amount present in dividing and non-dividing cells (O'Connor, 1953*b*).

This possible exception apart, the suggestion that the process of cell division depends on reactions leading to pyruvate formation can be regarded as additional to the dependence of cell division on carbohydrate metabolism for its

energy requirements. It may be that one or other dependence is limiting to cell division at different stages of the mitotic cycle, for Bullough & Johnson (1951) concluded that the respiratory oxidation of pyruvate is necessary for the initiation of cell division but not for its completion (see also Bullough, 1950). It might therefore be suggested that a continuance of cell division, once it has begun, depends upon reactions leading to pyruvate formation and in particular upon those associated with ribose-5-phosphate formation. Such a suggestion would be in accordance with the observations of Jacobson & Webb (1952) that ribonucleoprotein is formed in the nucleus during mitosis and extruded into the cytoplasm at anaphase. As mentioned above, such a formation of ribonucleic acid might, as an alternative, be possible from precursors already in the cell, and thus account for the ability of some cells to complete cell division, once it has begun, 'in almost any circumstances short of death of the cell itself' (Bullough, 1952).

If this suggested double dependence of mitotic activity on carbohydrate catabolism exists, cell proliferation might be controlled by a balance between the reactions leading to the formation of pyruvate and those of the tricarboxylic acid cycle. If the latter were limited by anaerobiosis an altered balance might cause a modification of proliferative capacity and in this way account for the association found by Goldblatt & Cameron (1953) between intermittent anaerobiosis and the malignant conversion of cultured fibroblasts. Again, it can be suggested that such a disturbed balance might occur if the respiratory mechanism of cells was damaged and replaced by a process involving lactic acid formation —a change that Warburg (1930) associated with the development of malignancy and one that might follow deficiencies in the tricarboxylic acid cycle for which evidence has been found in malignant tissue (Potter & Busch, 1950). Further, it might be possible to relate such a disturbed balance to changes in the cell fractions that can be separated by ultracentrifugation. It has been found that the reactions by which pyruvate is formed, whether by the glycolytic or the hexose monophosphate oxidative route, are associated with the soluble fraction (Le Page & Schneider, 1948; Glock & McLean, 1952), while the reactions of the tricarboxylic acid cycle are associated with the mitochondria (Harman, 1950).

SUMMARY

1. From micromanometric measurements made on liver tissue of the chicken embryo, isolated in a glucose-containing medium, estimates have been made of the rate of glucose catabolism ending in acid formation and that utilizing oxygen.

2. The variations undergone by these metabolic processes during normal development have been compared with the variations in mitotic activity of the hepatic cells as determined by the proportion of dividing cells seen in histological sections.

3. The comparison revealed two different associations in the embryonic liver

between carbohydrate metabolism and mitotic activity. Before the appearance of glycogen, mitotic activity, the estimated rate of acid production from glucose, and the rate of respiratory glucose utilization decreased in a corresponding manner. After the appearance of glycogen, mitotic activity persisted, but the estimated rates of both metabolic processes fell nearly to zero.

4. Both before and after the appearance of glycogen these relationships of mitotic activity to carbohydrate metabolism differed from those previously recorded for the midbrain and the red-blood cells, which in turn differ one from the other. The suggestion has been made that these differences are consistent with a common dependence of mitotic activity on metabolic reactions leading to pyruvate formation, special consideration being given to the reactions of the hexose monophosphate oxidative route (Dickens, 1953), concerned in the formation of ribose-5-phosphate.

5. This possible dependence is considered to be additional to a dependence of mitotic activity on the energy produced from carbohydrate by the reactions of the tricarboxylic cycle. The implications of such a double dependence of mitotic activity on carbohydrate metabolism have been discussed.

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An Electron Microscope Study of the Cartilaginous Matrix in the Developing Tibia of the Fowl

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WITH FIVE PLATES

INTRODUCTION

EXISTING accounts of structural changes in the developing long bones are based on histological and histochemical work, and their scope is limited by the resolving power of the optical microscope. The history of the cells involved in the formation and subsequent ossification of cartilage and the accompanying changes in the pattern of the tissue as a whole have been extensively studied (for literature see Fell, 1925). The intercellular matrix of embryonic cartilage, however, is less accessible to light optical methods than the cells and its fine structure is therefore incompletely known. The earliest investigators thought it to be homogeneous ('hyaline' cartilage), but later, with the improvement in microscopical techniques, a fine fibrous network was demonstrated which permeates the matrix and is generally held to be collagenous. This network becomes denser as development progresses. When the avian bones are completely ossified, only a thin layer of cartilage remains on the articular surfaces. This articular cartilage is intimately associated with the collagenous material of the joint capsules and the tendons, and itself contains coarse fibres.

In the present investigation an attempt has been made to study the fine structure of the cartilaginous matrix of a developing long bone, viz. the tibia of the fowl, by means of electron microscopy, and to correlate the results with those of ordinary histology. Thus, stained sections have proyided a 'map' of the tissue while corresponding thin sections and squash preparations, examined in the electron microscope (E.M.), supplied the fine-structural detail. Preliminary results of this work are reported briefly elsewhere (Martin, 1953).

TERMINOLOGY

To avoid confusion with the terminology applied to mammalian long-bones a definition is given below of the terms used in the present paper to specify certain regions of the developing fowl tibia (Text-fig. 1, A and B). The definitions

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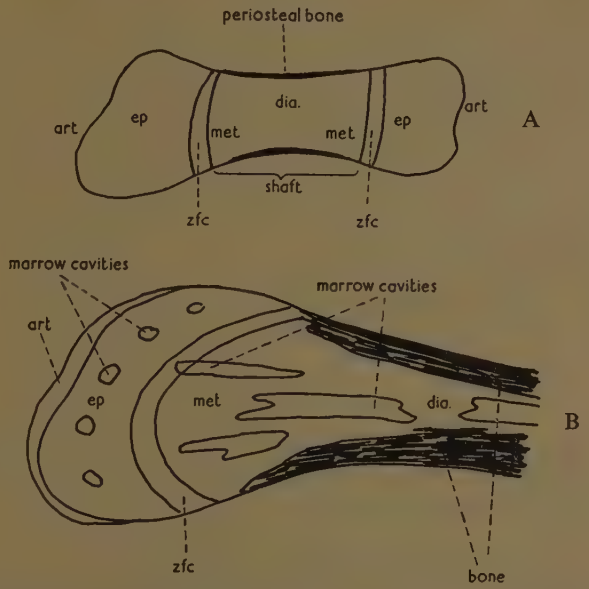
are based on Fell's work on the development of cartilage and bone in the fowl (Fell, 1925), where controversial issues such as that of the epiphyses in birds are discussed in detail.



TEXT-FIG. 1. The distribution of cartilaginous zones in the developing fowl tibia (diagrammatic). A. After 10 days' incubation. B. 1 day after hatching.

Articular cartilage (*art*): thin cartilaginous layer on the articular surface, continuous with the joint capsule and with tendons inserted in this region. This is the only part of the original cartilaginous precursor of the long bone which persists throughout the adult life of the animal.

Epiphysis (*ep*): region corresponding to the epiphysis of the mammalia but without an independent centre of ossification. Sometimes also referred to as 'small-cell region'.

Zone of flattened cells (*zfc*): region corresponding to the 'proliferative zone', 'epiphysial cartilage', or 'epiphysial plate' of the mammalia.

Metaphysis (*met*): region of the shaft adjoining the zone of flattened cells. The distinction between this part of the shaft and its centre is one of convenience only.

Diaphysis (*dia*): central part of the shaft.

MATERIAL AND METHODS

The material used for investigation in the optical and electron microscopes and the methods of preparation for the E.M. are summarized in Table 1.

All specimens were dissected in Tyrode's saline solution and fixed in a 2 per cent. buffered solution of osmium tetroxide at pH 7. The time of fixation varied between 5 minutes for small objects and 15–20 minutes for post-embryonic and

TABLE 1

	Age	art	ep	zfc	met	dia
Embryonic	5½ days	Squash of whole cartilaginous rudiment				
	7–12 days	Sections and squashes		Sections and squashes		—
	13–19 days	Sections and squashes	Sections and squashes	Sections and squashes	—	—
Post-embryonic	1 day	,,	,,	,,	Squashes	—
	2 days	,,	,,	,,	,,	—
	4 days	,,	,,	,,	,,	—
	7 days	,,	,,	,,	,,	—
	14 days	Squashes	Squashes	Squashes	,,	—
	21 days	,,	,,	,,	—	—
	Adult	,,	—	—	—	—

adult material. Fixation was followed by thorough washing under running water and three changes of glass-distilled water; if not used immediately the tissue was stored in 70 per cent. alcohol. Severely blackened specimens were bleached by short immersion in 30-vol. hydrogen peroxide.

'Squash' preparations for the E.M. were made by fragmenting the tissue with two fine scalpels or needles in a drop of clean glass-distilled water in a well slide. Drops of the resulting suspension were then transferred to formvar-coated specimen grids and, when dry, shadowed with palladium-gold for examination in the E.M. For sectioning, the tissue was dehydrated in the usual way and then treated according to the following schedule: (1) absolute alcohol overnight; (2) two hours each in butyl alcohol : chloroform 3 : 1, 1 : 1, 1 : 3; (3) chloroform overnight; (4) transferred to thermostat oven at 56° C., small quantities of ester wax being added at half-hourly intervals; (5) ester wax overnight; (6) some hours in fresh ester wax; (7) second change of ester wax and embedded.

Corresponding sections were cut for optical microscopy on a rocking microtome and for electron microscopy on a thin-sectioning machine designed and built in this department. Histological sections were stained with Celestin Blue Mayer–Van Gieson, or metachromatically with toluidin blue or methylene blue. The corresponding thin sections for electron microscopy were picked up on formvar-coated films and the ester wax removed by dropping xylene on to the sections and sucking it off again with the point of a triangular piece of filter-paper without touching the specimen or the supporting film. This process was repeated 3–4 times and the sections were then thinly shadowed with palladium-gold. After examination in the E.M. the sections were preserved on their supporting grids by mounting in DePex between a slide and coverslip; they were thus available for further study in the optical microscope if required.

In embryonic material the regions indicated in Text-fig. 1A are difficult to separate, and squash preparations of such specimens usually contain at least two cartilaginous zones, but in thin sections for electron microscopy the different regions are easy to identify so that micrographs of known and selected areas can be taken. In post-embryonic tibiae, on the other hand, the zones are easily distinguishable with the naked eye and can be dissected without difficulty, so that squash preparations can be made of each zone separately. This is important as thin sections of older undecalcified material are seldom entirely satisfactory on account of its brittleness and most information has to be obtained from the examination of squashes.

It was found convenient to divide the specimens studied into a number of age-groups representing successive characteristic stages of development. This grouping is, of course, arbitrary, since the development of the long bones is a continuous process. The age-groups to be discussed with respect to the fine structure of the cartilage matrix, and a brief indication of their histological characteristics, are given in Table 2.

TABLE 2

	Group	*Age (in days)*	*Histological characteristics of tibia*
Embryonic	I	5–7	Solid cartilaginous rod; no ossification; no marrow. Beginning hypertrophy of chondroblasts in diaphysis. Zone of flattened cells recognizable, but no sharp dividing line between epiphysis and shaft. Perichondrium only around diaphysis. Tissue of epiphysis continuous with surrounding connective tissue.
	II	8–13	Periosteal ossification of diaphysis. Perichondrium as far as articular cartilage. Main marrow cavity in shaft; smaller ones invading epiphysis. Sharp division between shaft and epiphysis.
	III	14–19	Continued ossification of shaft. Cells in zone of flattened cells and epiphysis retain their general appearance, but the zones themselves become narrower as they are 'pushed' away from the centre of the tibia towards the respective articular ends.
Post-embryonic	IV	1–17	The process of 'narrowing down' the cartilaginous areas of the head continues until
	V	8–21	only a thin layer of cartilage is left; epiphysial and flattened-cell portions of this layer still recognizable.
	VI	Adult	Tibia entirely ossified, only thin layer of articular cartilage remains.

RESULTS

Group I (5–7 days' incubation). At this stage the matrix in the articular and epiphysial regions appears devoid of fibres in the optical microscope, but with the aid of Van Gieson's picric acid-fuchsin stain faint fibre-bundles can be detected between the flattened cells; these become more distinct towards the centre of the shaft where the cells of the diaphysis are just beginning to hypertrophy.

In the E.M., however, squash preparations are shown to consist almost entirely of fine, apparently structureless, fibrils which form a dense feltwork (Plate 1, fig. B). These fibrils are long and of very constant diameter (about 400 Å); they are the only type of fibre present. In thin sections the feltwork

appears denser and more closely woven, and is uniformly distributed throughout the intercellular spaces. The 'fibres' seen in the optical microscope after staining with Van Gieson represent in reality accumulations of bundles made up of fine 'unbanded' fibrils; even the bundles would not be visible individually in the light microscope.

Group II (8–13 days' incubation). In this group periosteal bone-formation has begun; the fibrous perichondrium now extends as far as the articular region and the epiphysis is being invaded by small marrow cavities. The epiphysis is clearly defined and has a great affinity for metachromatic stains, while the zone of flattened cells gives an intense reaction with Van Gieson's picric acid-fuchsin. A fibrous element can now be demonstrated in the matrix of the epiphysis (Plate 1, fig. A), especially in the neighbourhood of the perichondrium and of the invading marrow cavities. These fibres are generally thought to be collagen, but electron micrographs of the epiphysis and the articular cartilage (Plate 1, figs. C and D) show a dense mass of fine, apparently unbanded fibrils similar to that previously demonstrated in squash preparations and sections of material of Group I. Very occasionally these fibrils appear to show a faint periodic banding of about 170 Å, but this was observed in squash preparations where the granularity of the background (due to the interfibrillar substance) sometimes creates the impression of a regular pattern. The continued use of the term 'unbanded' fibrils seems, therefore, justified at the present stage. In thin sections the fibrils are shown to be intricately interwoven and looped around one another and their straightened appearance in squash preparations is due to mechanical separation and subsequent drying; there is no obvious tendency to become orientated into parallel bundles or to form denser capsules around the cells. Occasionally coarser fibres with periodic cross-striations of 400 Å can be observed, mainly in the neighbourhood of cells, but neither sections nor squashes indicate the origin of these fibres and their relationship with the 'unbanded' fibrils and the cells. Of the many squashes and thin sections of cartilage from the epiphysis and articular region of this age-group examined in the E.M., none contained fibres with the periodic cross-banding characteristic of mature collagen (670–90 Å in the fowl). On the other hand, periosteal bone from corresponding tibiae, when fragmented mechanically and prepared for electron microscopy in the manner described, yields collagen fibres of the mature type (Plate 2, fig. G); these fibres are usually coated with bone salts, but their cross-banded structure is plainly visible. Cartilage of the metaphysis also contains some fibres with the repeating pattern characteristic of mature collagen. Though relatively rare, these fibres are easily recognizable among the mass of thin 'unbanded' fibrils (Plate 2, fig. E); they differ from fully mature collagen only in that they are short and have tapering ends, thus resembling collagen fibres reprecipitated from solutions (Vanamee & Porter, 1951).

Group III (14–19 days' incubation) includes well-ossified tibiae up to the time of hatching. In the youngest specimens of this group the shaft already consists

of a stout tube of trabecular bone, a fibrous perichondrium surrounds the entire epiphysial and articular region, and the epiphysis has been invaded by numerous marrow cavities surrounded by densely fibrous material (Plate 3, fig. J). In the epiphysis the cartilage matrix still exhibits a much more intense metachromasia than the diminishing cartilage of the other zones, while the cells remain small and tightly packed and show no sign of hypertrophy except in the oldest specimens of the group. The fibrous component of the cartilage matrix is now very prominent even in the epiphysis, the thickest and most intensely staining fibre-bundles being found in the neighbourhood of the perichondrium from which they appear to originate (Plate 2, fig. F).

In the E.M. the perichondrium can be shown to contain relatively few, rather elongated cells; it seems to consist almost entirely of fibres having a somewhat variable periodic structure differing from that of mature collagen (Plate 2, fig. H). In some fibres bands of equal density are spaced about 270 Å apart; in others the density varies with every other or every third band, the distance between the more prominent bands being about 550 Å. Sometimes a twin-banded structure similar to that of mature cartilage is observed, but the total period only measures about 550 Å as compared with the 670–90 Å of mature fowl collagen. Bundles of these fibres can be traced some distance into the cartilage matrix, where they are eventually lost in the network of fine fibrils which is still present and which closely resembles that of much younger specimens. Despite this similarity, however, important differences between the structure of the matrix in younger and in older cartilage are found. Thus the fine fibrils no longer form mainly a diffuse feltwork which fills the spaces between the cells, but oriented bundles have appeared in which individual fibrils are tightly packed together and in a definite direction which is usually related to the neighbouring cartilage cells (Plate 3, figs. K and L); some fibre-bundles appear to be 'avoiding' the cells, steering an almost straight course through the intercellular matrix while maintaining roughly the same distance from any cells encountered on the way, while other bundles run concentrically around individual chondroblasts as though beginning to form fibrous capsules. In none of these fibre-bundles can the cross-banding of mature collagen be demonstrated.

In the immediate neighbourhood of marrow cavities the cartilage is being eroded. The tissue is torn and the cells retracted, and exposure to the electron beam tends to exaggerate this condition by enlarging the tears and causing new ones. Thin sections through such eroded areas (Plate 3, fig. I) show that, although distorted and under tension, the fine fibrous network of the matrix does not differ materially from that in other parts of the epiphysis. The strong staining reaction with Van Gieson observed in these areas (Plate 3, fig. J) does not appear to be correlated with the presence of mature collagenous fibres or with a marked accumulation of oriented bundles within the limits of thickness of the thin sections examined. This does not mean that no such accumulation exists, but that it may be visible only in the thicker sections used for optical work.

Groups IV and V (1–21 days post-embryonic). The replacement of immature banded fibres in the articular region (Plate 2, fig. H) by fibres easily recognizable as mature collagen (Plate 4, fig. N) takes place in the tibia at the time of hatching or shortly after (Plate 4, fig. M) and is completed by the end of the first post-embryonic week. The age at which this transformation begins cannot be given accurately as there seems to be great individual variation. Thin, 'unbanded', fibrils similar to those demonstrated in the early embryonic tibia remain, but appear to be fewer in proportion to the increasing collagen content of the cartilage. The long, banded fibres—whether mature or immature—are gathered into massive bundles and short, isolated collagen-fibres, such as those found in the metaphysis of embryonic tibiae after only 11 days' incubation, do not seem to occur in the articular cartilage during any stage of development.

The macroscopically visible differences between the articular cartilage, epiphysis, and zone of flattened cells in the young chick (see Text-fig. 1B) correspond to differences in the fine structure of the matrix in these regions. These differences persist until all but the articular cartilage is ossified. After the first week of post-embryonic development the fibrous component of the articular cartilage (Plate 4, fig. N) consists of mature collagen and a relatively small proportion of 'unbanded' fibrils. The matrix of the epiphysis (Plate 5, fig. R) contains 'unbanded' fibrils, closely interwoven much as in embryonic cartilage, a large proportion of immature banded fibres, and long, isolated mature collagen-fibres. By the end of the third post-embryonic week the content of mature collagen fibres has increased noticeably and the matrix of the epiphysis now closely resembles that of the articular cartilage (Plate 5, fig. S). In the zone of flattened cells (Plate 5, fig. T), the amount of intercellular material present is outweighed by cells and cell debris, and the fine structure of the fibres is often obscured by amorphous material which renders accurate measurements difficult. It is possible, however, to identify fibres with the mature and immature type of cross-striation, and to distinguish them from the thin 'unbanded' fibrils.

Group VI. The optical microscope shows a close resemblance between the articular cartilage in the young chick (Plate 4, fig. P) and that in the adult fowl (Plate 4, fig. Q). This similarity also exists on the level of magnification of the E.M. A comparison between a squash preparation of young post-embryonic (Plate 4, fig. N) and adult (Plate 4, fig. O) material shows that in both well-defined collagen-fibres constitute the main bulk of the intercellular matrix, the only difference being that the average thickness of the collagen-fibres in the adult fowl is greater than in the young chick. 'Unbanded' fibrils are still present, and have been found in the articular cartilage even of very old birds.

DISCUSSION

The main facts emerging from the evidence presented above are: (1) that during embryonic and post-embryonic development the fibrous component of the cartilage matrix undergoes structural changes visible only in the E.M.; and

(2) that, depending on whether cartilage is being replaced by bone or developing into mature articular cartilage, these structural changes appear to follow a somewhat different pattern.

It has been shown that in the cartilage matrix of young embryos only one type of fibre is present which we have termed 'unbanded' fibrils as no banded structure can be observed in them with certainty. As the development of the articular region and the epiphysis progresses another type of fibre appears which exhibits what we have called the immature type of cross-banding. These fibres are finally replaced by or converted into others with the well-known periodic structure of mature collagen (670–90 Å in the fowl). 'Unbanded' fibrils persist throughout embryonic and post-embryonic development and are found in adult specimens, including very old ones.

The question arises whether these 'unbanded' fibrils are precursors of immature banded fibres, and these in turn of the mature collagen of the adult articular cartilage, or whether the two types of cross-striated fibre are related while the 'unbanded' fibrils exist independently. On the present evidence this question must remain undecided, but the second alternative would appear to be the more likely one. If the first assumption were correct, the presence of 'unbanded' fibrils in mature cartilage could only mean that new collagen-fibres are being formed continually from structurally different precursors, and that in this continual development the intermediate stage observed in younger material, i.e. fibres with the immature type of cross-banding, is omitted. It appears more reasonable to assume that the 'unbanded' fibrils are not precursors of collagen but independent and chemically different structures which precede and accompany the formation of immature banded fibres and their replacement by mature collagen.

The time at which collagen-fibres begin to appear in the tibia of the developing fowl varies in the different regions. It has been demonstrated (Plate 2, figs. E and F) that fibres with the mature type of cross-striation occur in the cartilage of the metaphysis and in the periosteal bone of the tibia after only 11 days' incubation; i.e. at a time when not even immature collagen-fibres are found in the epiphysis and articular region. This seems to indicate that collagen-fibres are in some way connected with ossification and make their first appearance in regions where bone is being formed or is about to be formed. The 'mature' fibres from the 11-day metaphysis, however, are rather short and have tapering ends; they differ therefore from the fully mature, long collagen-fibres of tendon and bone. In the articular region of the fowl tibia such short and tapering fibres cannot be found during any stage of development. Instead, long and thin fibres with cross-bands, which we have described as 'immature', begin to appear around the 13th or 14th day of incubation; these persist until after the young chick has hatched and are then gradually converted into, or replaced by, equally long and still rather thin fibres with the mature type of cross-banding. The collagen-fibres at sites of ossification in the shaft of the tibia thus appear to develop differently

from those in the articular zone. Superficially, the short, tapering fibres of the metaphysis resemble some fibres reprecipitated from collagen solutions (Vanamee & Porter, 1951), while the long, thin fibres with immature cross-banding are very similar to embryonic collagen from mammalian sources (Randall *et al.*, 1952), and to another type of reprecipitated material (Highberger *et al.*, 1951). In the epiphysis and the zone of flattened cells collagen develops more slowly than in the other regions, and although these parts of the tibia eventually ossify, the development here resembles more closely that in the articular cartilage than that in the metaphysis. The electron micrographs, Plate 5, fig. R and fig. T, show that in these areas long, immature and mature collagenous fibres occur together for some weeks after hatching; they usually appear rather isolated against a background of 'unbanded' fibrils. When ossification of the metaphysis is completed, the zone of flattened cells is the next site of bone formation, but the fine structure of the matrix here bears no resemblance to that of the metaphysis immediately prior to ossification. No explanation can be offered, but one might suggest tentatively that physiological differences of some kind exist between the tissues of the shaft, on the one hand, and those of the zone of flattened cells and epiphysis on the other. Thus the dividing line between the shaft and the head of the tibia may have a deeper significance than has been suspected hitherto. Ossification in the epiphysis is a slow process as compared with that in the shaft, and there may well be some functional relationship between the speed of ossification and the type of fibre present in the matrix of the cartilage about to become ossified. Beyond this the evidence obtained by the present method offers no clue as to the role played by the fibres of the cartilage matrix during bone development.

It remains to point out again that optical histology can give no indication of the complexity of the fine-structural changes involved in the development of the long bones of the fowl.

SUMMARY

1. The methods of optical and electron histology have been applied to the study of structural changes in the cartilaginous matrix of the developing fowl tibia.

2. A fine fibrous component without distinctly recognizable periodic structure occurs in the cartilage matrix throughout development and in adult life.

3. Collagen-fibres appear later than the 'unbanded' fibrils and develop differently in the different regions of the tibia.

4. The possible significance of these observations is discussed.

ACKNOWLEDGEMENTS

I am indebted to Professor J. T. Randall, F.R.S., for his interest and encouragement and to Dr. H. B. Fell, F.R.S., for much valuable discussion and

criticism. I wish to thank Mr. B. Richards for preparing the photomicrographs reproduced in Figs. A, F, J, O, and P.

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EXPLANATION OF PLATES

PLATE 1

FIG. A. Photomicrograph of a section through the tibia of a 10-day embryo stained with Van Gieson. The zone of flattened cells (*zfc*) shows the strongest staining reaction; it represents the dividing line between the small-celled epiphysis (*ep*) and the metaphysis (*met*) or external portion of the shaft. In the spaces between densely stained chondroblasts of the metaphysis a fibrous component is visible.

FIG. B. Electron micrograph of a section through the epiphysis of a 7-day embryonic tibia showing a chondroblast surrounded by the dense feltwork of 'unbanded' fibrils. Shadowed with Pd-Au. Magnification ×10,000.

FIG. C. Electron micrograph of a squash preparation of a 13-day embryonic tibial epiphysis showing 'unbanded' fibrils. Shadowed with Pd-Au. Magnification ×27,000.

FIG. D. Electron micrograph of a longitudinal section through the epiphysis of a 10-day embryonic tibia showing the absence of capsules or clear spaces around the chondroblasts. Some coarser fibres (*bf*) have cross-striations of about 400 Å; they occur rarely but usually in the neighbourhood of cells. 'Ridges' (*ri*) formed by the feltwork of fibres do not represent fibre-bundles, but might be mistaken for such if seen at lower magnification. Shadowed with Pd-Au. Magnification ×15,000.

PLATE 2

FIG. E. Electron micrograph of a squash preparation of an 11-day embryonic metaphysis showing short, tapering fibres with the periodic structure of mature collagen against a background of 'unbanded' fibrils. Shadowed with Pd-Au. Magnification ×22,000.

FIG. F. Photomicrograph of the epiphysis of a 14-day embryonic tibia in the immediate neighbourhood of the articular region (*art*). The intercellular spaces at the point of fusion with the articular cartilage appear dark owing to the reaction of the intercellular fibres with Van Gieson's stain; a less intensively stained fibrous component is visible between the chondroblasts of the epiphysis.

FIG. G. Collagenous fibre from the periosteal sleeve of a 12-day embryonic tibia; isolated by teasing the undecalcified material. Shadowed with Pd-Au. Magnification ×23,000.

FIG. H. Electron micrograph of 'immature' collagenous fibres from 19-day embryonic articular cartilage and articular perichondrium. Note the variability of the pattern of cross-striations. Shadowed with Pd-Au. Magnification ×15,000.

Plate 3

Fig. I. Electron micrograph of a section through the epiphysis of a 14-day embryonic tibia near an invading marrow cavity. The cells in this region are retracted and gaps have formed around them; one of these pericellular spaces has been cut open in this section. Coarser fibres like those found associated with chondroblasts in younger material (see Plate 1, fig. D) are found in the neighbourhood of the 'gap' and at one point (*bf*) their cross-striations are recognizable. Shadowed with Pd-Au. Magnification ×15,000.

Fig. J. Photomicrograph of a marrow cavity in a 14-day embryonic epiphysis. The walls of the cavity and the adjoining regions stain very darkly with Van Gieson as though containing much collagenous material.

Fig. K. Electron micrograph of a section through the epiphysis of a 19-day embryonic tibia; showing increasing orientation of fibre-bundles. Magnification ×15,000.

Fig. L. Electron micrograph of a section through a 14-day embryonic epiphysis showing the transition from perichondrium to cartilage. The elongated cell showing on the left is a cell of the perichondrium, but coarse fibres associated with it are extending well into the cartilaginous matrix. Magnification ×15,000.

Plate 4

Fig. M. Electron micrograph of a section through the articular cartilage and perichondrium of a 1-day post-embryonic chick showing long, twisted bundles made up of immature collagenous fibres. Shadowed with Pd-Au. Magnification ×18,000.

Fig. N. Electron micrograph of a teased preparation of articular cartilage from a 1-week post-embryonic chick; the bundles shown consist mainly of fibres with the cross-banding of mature collagen. Shadowed with Pd-Au. Magnification ×18,000.

Fig. O. Electron micrograph of a teased preparation of adult articular cartilage. Mature collagen-fibres of varying thicknesses are present, as well as 'unbanded' fibrils. Shadowed with Pd-Au. Magnification ×28,000.

Fig. P. Photomicrograph of a section through the articular cartilage of a young chick. The fibrous component (*cf*) is now very prominent and stains very intensively with Van Gieson.

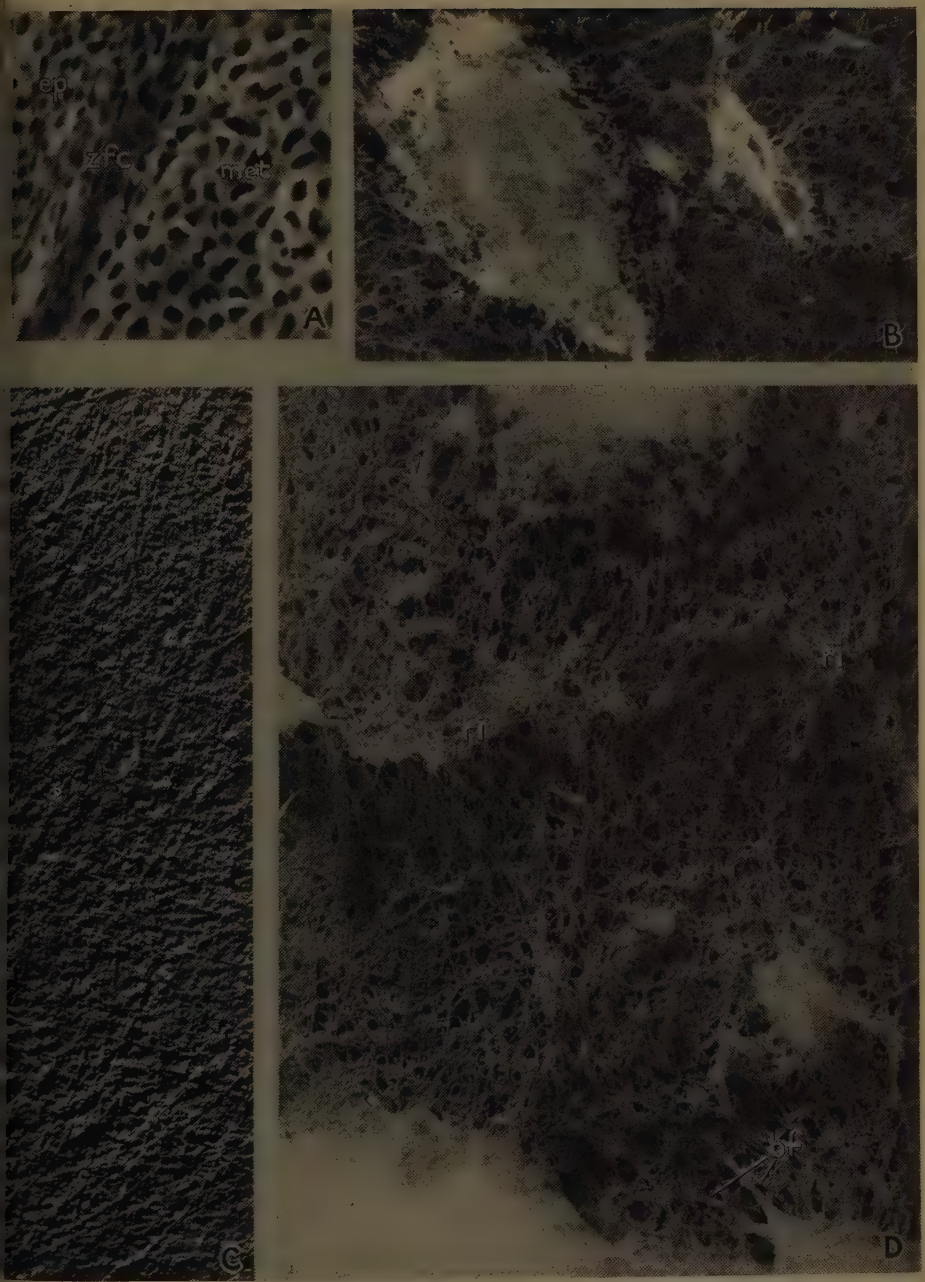
Fig. O. Photomicrograph of a section through adult articular cartilage showing an array of fibre-bundles similar to that found in the young chick, but distributed over a wide area.

Plate 5

Fig. R. Electron micrograph of a squash preparation of the epiphysis of a 1-week post-embryonic tibia. Mature collagen-fibres and 'unbanded' fibrils can be clearly distinguished. Shadowed with Pd-Au. Magnification ×18,000.

Fig. S. Electron micrograph of a squash preparation of the epiphysis of a 3-week post-embryonic tibia. The two biggest collagen-fibres in the centre show 'sub-bands'. Shadowed with Pd-Au. Magnification ×15,000.

Fig. T. Electron micrograph of a squash preparation of the zone of flattened cells of a 1-week post-embryonic tibia. Cell debris obscures the fibres, but the 'immature' type of banding can be recognized in some of them (*bf*). Shadowed with Pd-Au. Magnification ×18,000.



A. V. W. MARTIN

Plate 1

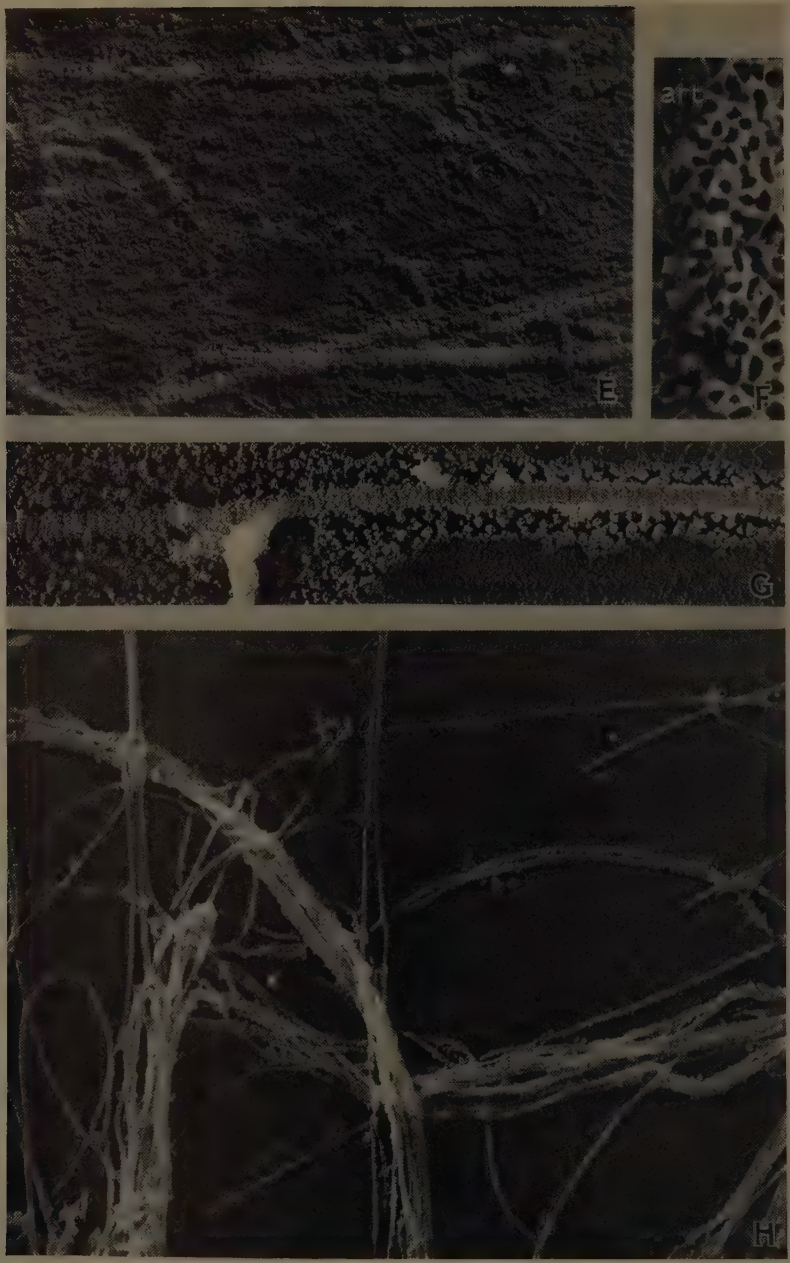


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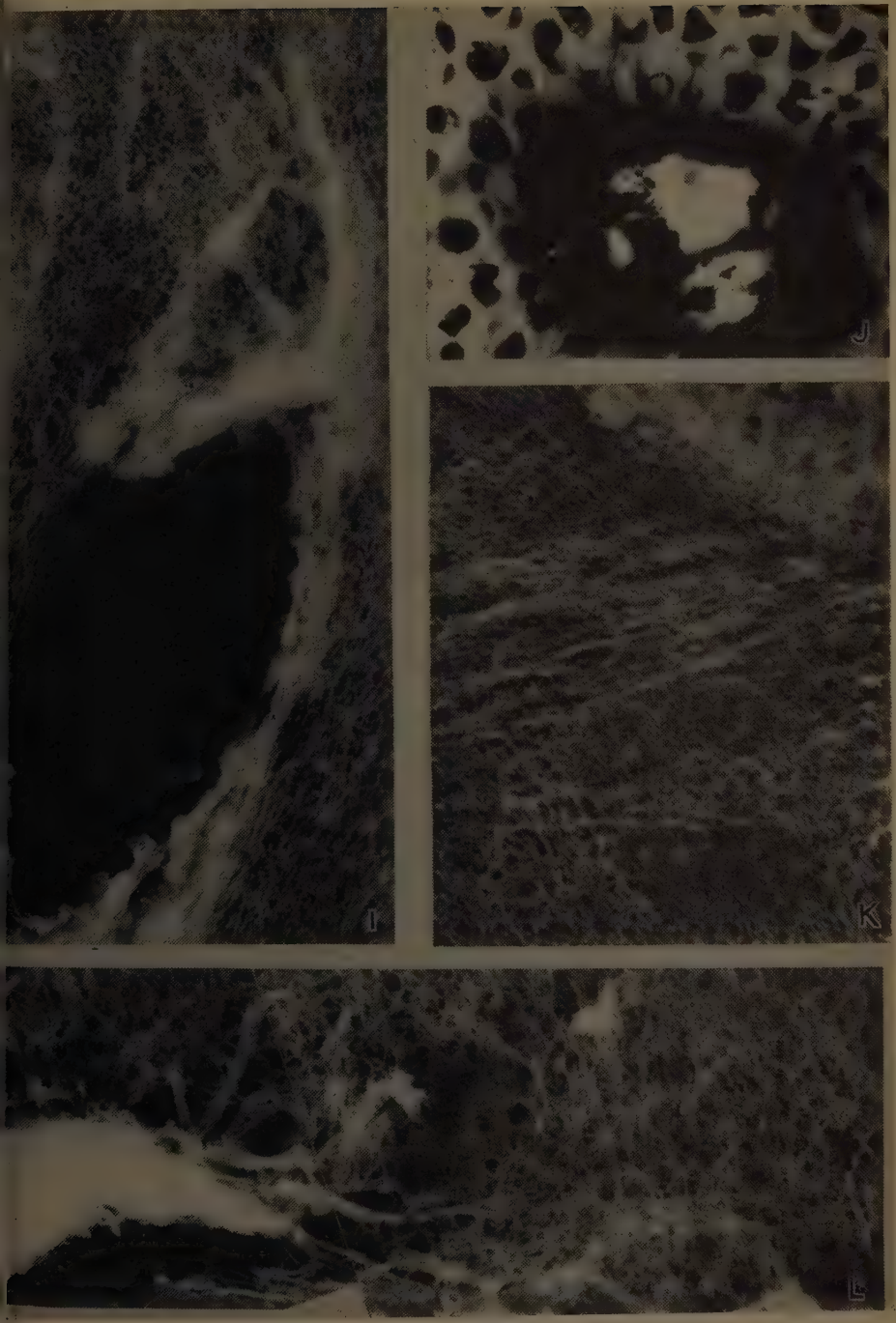
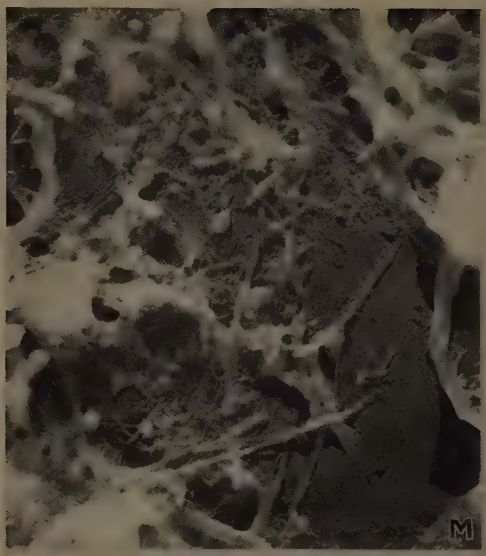
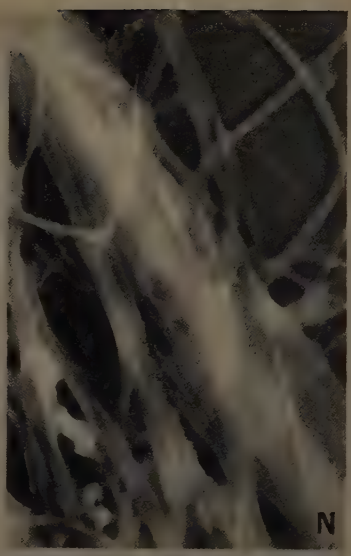
Plate 2

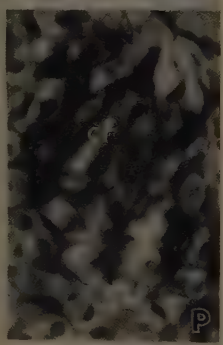


A. V. W. MARTIN

Plate 3

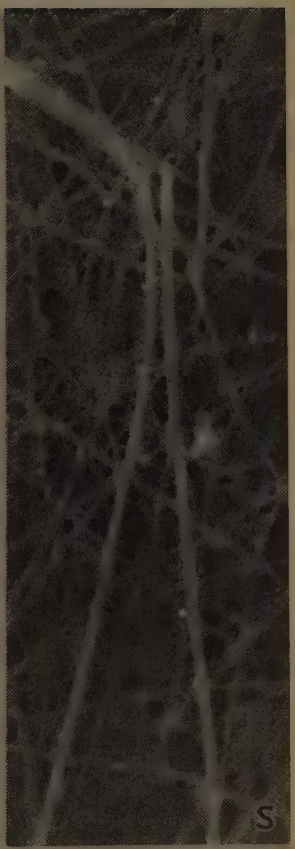


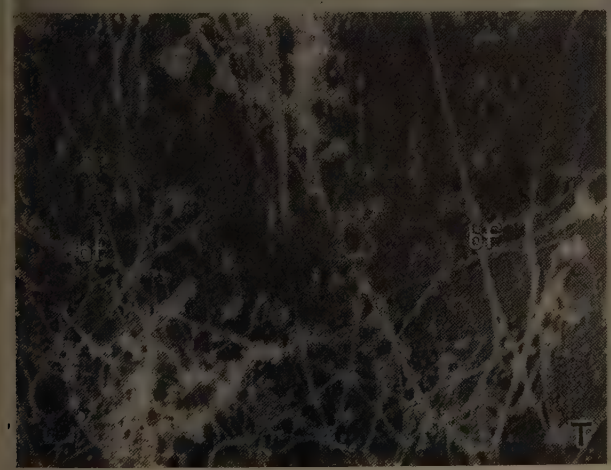




A. V. W. MARTIN

Plate 4





A. V. W. MARTIN

Plate 5

Size of Fragment and Rate of Regeneration in Planarians

by AGNES *and* H. V. BRØNDSTED[1]

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INTRODUCTION

THE splendid investigations by Wolff & Dubois (1948) and Dubois (1949) on the cellular material concerned in the regeneration of planarians have again raised the question whether the process of regeneration follows the course of a mass-reaction, as maintained long ago by Loeb. Dubois (1949) has shown conclusively that the neoblasts (totipotent regeneration cells) in the regeneration blastema may have wandered to the wound from faraway regions of the body. The question as to whether the number of cells determines the speed of regeneration can therefore be approached by experiments relating size of the regenerating piece to speed of regeneration. On this point, conflicting statements are to be found in the literature. No clear distinction has, however, been made between two separate problems: the time of first appearance of the regenerated organs and the size of the organ-anlagen at this time; or, put in another way, the speed of determination of the neoblasts and the number of neoblasts available for determination. Only the first problem has been investigated here; the latter will be dealt with in a paper in preparation.

Abeloos (1930) found that, in *Planaria gonocephala*, in animals of about the same age the rate of regeneration of eyes from a given level of the antero-posterior axis is the same regardless of the size of the regenerating fragment. He also found that young and small individuals regenerate eyes faster than bigger and older ones. Abeloos holds that age or other physiological conditions and not merely size of regenerating fragment is important for the rate of regeneration. Buchanan (1933) also reported that small segments regenerate at the same rate as big ones; whereas Scharoff (1934) observed that small segments regenerate faster than bigger ones.

A reinvestigation of the relation of size of regenerating piece to speed of regeneration has been made with four planarian species: *Dendrocoelum lacteum*, *Bdellocephala punctata*, *Euplanaria lugubris*, *Polycelis nigra*.

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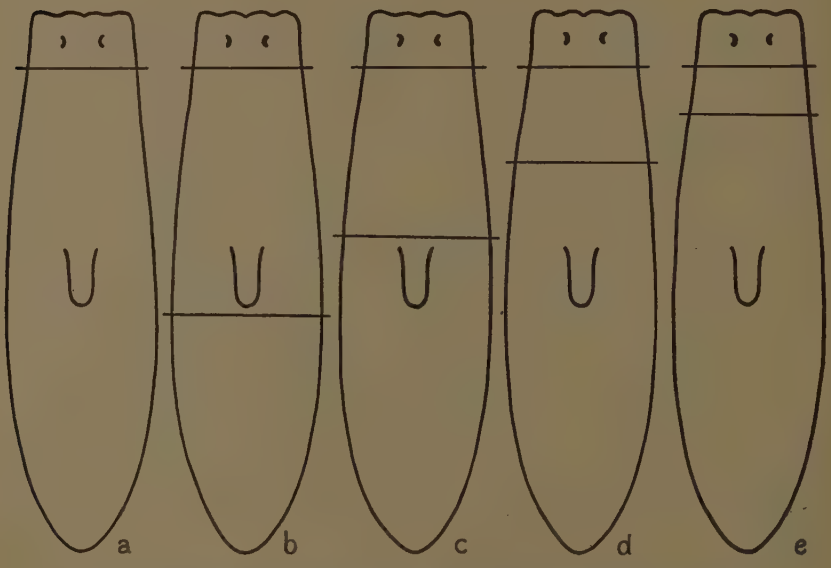
MATERIAL AND METHOD

The animals were captured under stones on the shores of Lake Furesø near Copenhagen. The experiments were carried out in a uniform way. The animals were kept in Petri dishes containing 50 ml. of tap-water at room temperature. Each dish contained 10 animals, the water was changed every day, and there was no feeding. Regeneration was regarded as having taken place when eye spots could be seen at a magnification of 25 times with a standard illumination.



TEXT-FIG. 1. *Dendrocoelum lacteum.* Five types of operation. In all, the head was removed by an anterior cut. In *b–e*, varying amounts of the posterior end of the body were removed by a second cut.

TABLE 1

Percentage regeneration of Dendrocoelum lacteum

Hours of regeneration	*Type of operation*				
	a	*b*	*c*	*d*	*e*
96	11	13	7	7	10
117	88	86	77	83	80
140	100	100	100	100	100

The types of operation are shown in Text-fig. 1. Thirty animals in each group.

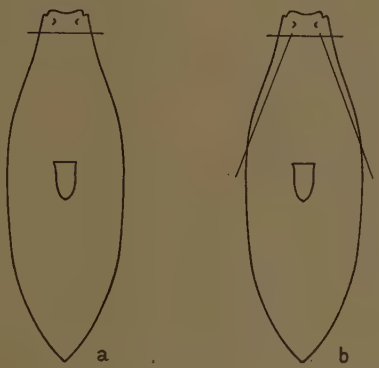
RESULTS

Dendrocoelum lacteum

One hundred and fifty animals were operated on, 30 animals in each of the five ways indicated in Text-fig. 1. The results are given in Table 1. Regeneration starts simultaneously and is terminated at the same time after all five types of operation. The rate of regeneration is therefore independent of the size of the regenerating piece.

Bdellocephala punctata

Thirty animals were cut as indicated in Text-fig. 2, operation *a*, and 30 as in



TEXT-FIG. 2. *Bdellocephala punctata.* Two types of operation.

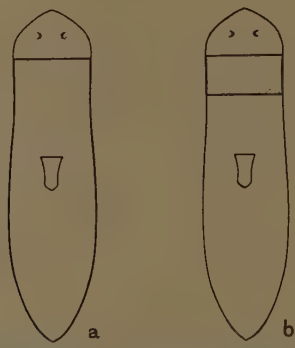
Text-fig. 2, operation *b*. This experiment was performed to see whether severe wounds calling upon a great number of neoblasts might retard the regeneration of eye spots. The results indicate that this is not the case. After 133 hours both groups show 33 per cent. regeneration. After 154 hours the figures were 70 per cent. after type *a* operation and 63 per cent. after type *b*. After 179 hours all living animals, 28 in each group, had reached 100 per cent. regeneration.

This experiment therefore shows that the rate of regeneration is not affected by increasing the severity of wounding in this way.

Euplanaria lugubris

Eighty animals were cut as indicated in Text-fig. 3, *a*. The anterior cut was made behind the eyes so that the distance from the cut to the eyes was the same as the distance from the eyes to the front end of the animal. Forty of the animals were again cut as indicated in *b*, the segments thus cut off ('*b*-pieces') being of the same length as the separated heads; the *b*-pieces were very short in com-

parison with the decapitated animals left after operation *a* ('*a*-pieces'), with correspondingly fewer neoblasts. Furthermore, whereas the *a*-pieces only regenerate heads, the *b*-pieces also regenerate tails from the caudal wound, laying therefore a heavier tax on their neoblasts. Nevertheless, the rate of eye regeneration was the same in *a*-pieces and in *b*-pieces. After 116 hours all animals in both groups had regenerated eyes. The size of eyes and of regeneration blastemas is larger, however, in the *a*-pieces. Detailed data on size differences will be given in a subsequent paper.



TEXT-FIG. 3. *Euplanaria lugubris*. Two types of operation. In *a*, the head is removed. In *b*, the posterior part of the body is also removed by a second cut.

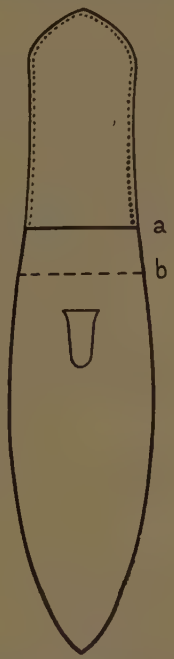
Polycelis nigra

This planarian is characterized by a string of eyes just within the border of the body in its anterior third. The head frequency curve has been worked out for the population in Lake Furesø near Copenhagen (Brøndsted, 1942). The time-graded field has not yet been studied in detail, but in analogy with *Bdellocephala punctata*, *Dendrocoelum lacteum*, and *Euplanaria lugubris* it seems safe to conclude that the head frequency curve coincides with the antero-posterior axis of a time-graded field in *Polycelis*; that this is so has been partly shown (unpublished). If this conclusion holds, the time required to regenerate eyes increases from the fore part of the body to the hind end. It is therefore imperative to make the transverse cut at a standard level, and this can best be done at the level just behind the eye string, which was accordingly used in the present experiments.

If the regeneration from the anterior surface of the transverse cut proceeds normally, the first appearance of eyes occurs on the sides of the base of the more or less conical regeneration blastema (Text-fig. 4*a*). In several animals, especially short pieces, the wound contracts in the manner indicated in Text-fig. 4*b*. In these no regeneration takes place, the probable explanation being, as pointed out by one of us (Brøndsted, 1939), that the anteriorly oriented gradients from both sides of the body oppose one another and thus mutually inhibit the formation of blastemata.

Eighty animals were operated upon. A transverse cut was made just behind the eye string, as shown by *a* in Text-fig. 5. Forty of these animals constitute group *a*. In the 40 remaining animals, making group *b*, the posterior end of the body was also removed by a second transverse cut 0·5 mm. farther back, at *b* in Text-fig. 5. Six days after the operation the eyes began to appear. The number

TEXT-FIG. 4. *Polycelis nigra.* (*a*) First appearance of eyes on regeneration blastema. (*b*) Wound contraction, which inhibits regeneration.



TEXT-FIG. 5. *Polycelis nigra.* Operation types. Either deletion of the head by cut at *a*, or deletion of both head end and posterion end by cuts at *a* and *b*.

of individuals with eye spots regenerated were counted 145 hours after operation. In group *a*, 34 individuals had survived, and 23 (68 per cent.) had regenerated eyes. In group *b*, only 18 specimens survived, but 15 (83 per cent.) had regenerated eyes. It can therefore be concluded that in *Polycelis* also the size of the regenerating body segment is not important for the rate of regeneration.

DISCUSSION

Dubois (1949) has conclusively shown that neoblasts forming the blastema are derived not only from neighbouring regions of the wound but also, if necessary, from distant parts of the body. It is tempting to conclude that the rate of

regeneration is dependent on the number of neoblasts wandering to the wound. But if that were the case one would expect to find a difference in the rate of regeneration in large and small fragments. We did not do so. The assumption is made further improbable by the fact that short transverse fragments (in addition to providing for the head-forming blastema) also provide neoblasts for the regeneration of the hinder end of the body.

The experiments were done on four different species, none of which propagate by fission. Our results are not therefore influenced by factors arising from physiological processes due to asexual formation of new individuals, as for instance is the case in *Planaria dorotocephala*. We consider that the species here used furnish more reliable results than do species with fission.

Only when, as in the experiments of Dubois (1949), the neoblasts in the piece which is to regenerate are killed by X-rays is regeneration retarded. This is because no neoblasts are immediately available for blastema formation; they have to migrate from distant non-irradiated parts of the body.

SUMMARY

Neither size of regenerating piece nor extent of wound influences rate of regeneration of eyes from a given level of the body in four species of planarian.

Our thanks for financial support are due to the Carlsberg Foundation, Nordisk Insulin Foundation, and Kongstad Foundation.

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The Effects in Chick Blastoderms of replacing the Primitive Node by a Graft of Posterior Primitive Streak

by M. ABERCROMBIE *and* RUTH BELLAIRS[1]

From the Department of Anatomy and Embryology, University College London

WITH ONE PLATE

INTRODUCTION

THE demonstration by Waddington (1932 and later) that the chick primitive streak has some of the properties of the amphibian 'organization centre' makes detailed investigation of its functions in development a matter of particular interest. A useful method of analysis is the exchange of parts within the streak. Abercrombie (1950) found that such exchange is technically possible, and he investigated the effects of reversing the antero-posterior polarity of pieces of the streak. The present investigation is concerned with another kind of transplantation within the streak: the replacement of its anterior end by a piece from its posterior end. The experiments described here were all made on blastoderms with a full-length or almost full-length primitive streak. Their main result was the production of numbers of embryos with anterior axial duplication. Several variants of the kind of operation used in the present work have been tried in order to carry the analysis farther. They will be reported in a subsequent paper.

METHOD

The tissue-culture technique of Waddington (1932) was used. Operations were performed with fine steel knives. In removing the anterior piece of the primitive streak, a square consisting of the whole primitive node (as defined by Wetzel, 1929) with primitive pit and underlying endoderm was cut out. Its edges coincided with the superficially visible margins of the node. The length of the side of the eliminated square was between 190μ and 280μ (average 230μ). In control specimens the excised node was simply replaced. In the other specimens the hole was filled with a graft similarly cut from within the posterior third of the primitive streak, usually of the same blastoderm. The dorso-ventral orientation of the graft when transplanted conformed with that of the rest of the

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blastoderm; but with a few exceptions its antero-posterior orientation was not controlled, in view of the rarity of any effect of reversing lengths of the streak (Abercrombie, 1950). The posterior hole was left unfilled and usually healed; but when it did not heal it caused no interference with development of the embryonic axis. Separate host and donor embryos were used in some operations, including a number where host and graft were made distinct by marking one of them with phosphorus[32] (Abercrombie & Causey, 1950). Embryos were grown *in vitro* at 38° for 24 hours after the operation unless otherwise stated. They were fixed in Bouin's fluid and serially sectioned at 10μ.

MATERIAL

The blastoderms at operation were of L or LM stage on Waddington's scale (see Abercrombie, 1950). Five embryos were fixed and sectioned immediately after deletion of the node. In all these embryos mesenchyme lay anteriorly to the hole, extending 200μ–400μ (average 250μ) forwards. There was also mesenchyme at either side of the hole, extending 100μ–1,000μ (average 500μ) laterally. This disposition of mesenchyme is in accordance with the descriptions of Wetzel (1929) and Adelmann (1922).

The presumptive fate of the tissue removed by the deletion of the node, according to Wetzel (1929), Wolff (1936), Pasteels (1937), and others, is notochord and floor of the neural tube. Spratt (1952) indicates, however, that little presumptive neural tissue would be included. Its endoderm is part of the presumptive foregut (Bellairs, 1953*a*). The mesoderm remaining anterior to the hole after the excision is mostly presumptive prechordal mesoderm, dorsal to which there lies the presumptive prechordal neural plate, i.e. fore-brain (Wetzel, 1929; Stein, 1933). The mesoderm lateral to the hole, according to Pasteels, is presumptive side plate or somite. It probably also includes presumptive heart (Rudnick, 1944) and perhaps presumptive parachordal head mesenchyme, the disposition of which is not clear from Pasteels's account. Immediately behind the hole a little presumptive notochord may remain after the operation (Wetzel, 1929; Gräper, 1929; Waddington, 1932; Spratt & Condon, 1947). Pasteels (1937) suggests that the notochordal material is invaginated through the part of the streak just behind the node, and some operations may have been done before the invagination was quite completed. It is completed before regression of the node begins (Spratt, 1947).

The graft at the time of operation consisted of loose mesenchyme, a certain amount of epiblast, and a layer of endoderm (the region is described by Wetzel, 1929). The presumptive fate of its mesoderm is side-plate and blood-island (Pasteels, 1937).

The stage at which operations were done is soon followed, in normal development, by the movement of extension which leads to axis formation. The node becomes drawn out, most of its material moving towards the posterior end of the blastoderm; and the more laterally situated presumptive axial mesoderm

and the rest of the presumptive neural plate also extend backwards (Wetzel, 1929; Pasteels, 1937). Some forward movement of presumptive anterior notochord (Spratt, 1947), of presumptive foregut (Bellairs, 1953*b*), and probably of presumptive prechordal mesoderm and prechordal neural plate also occurs.

RESULTS

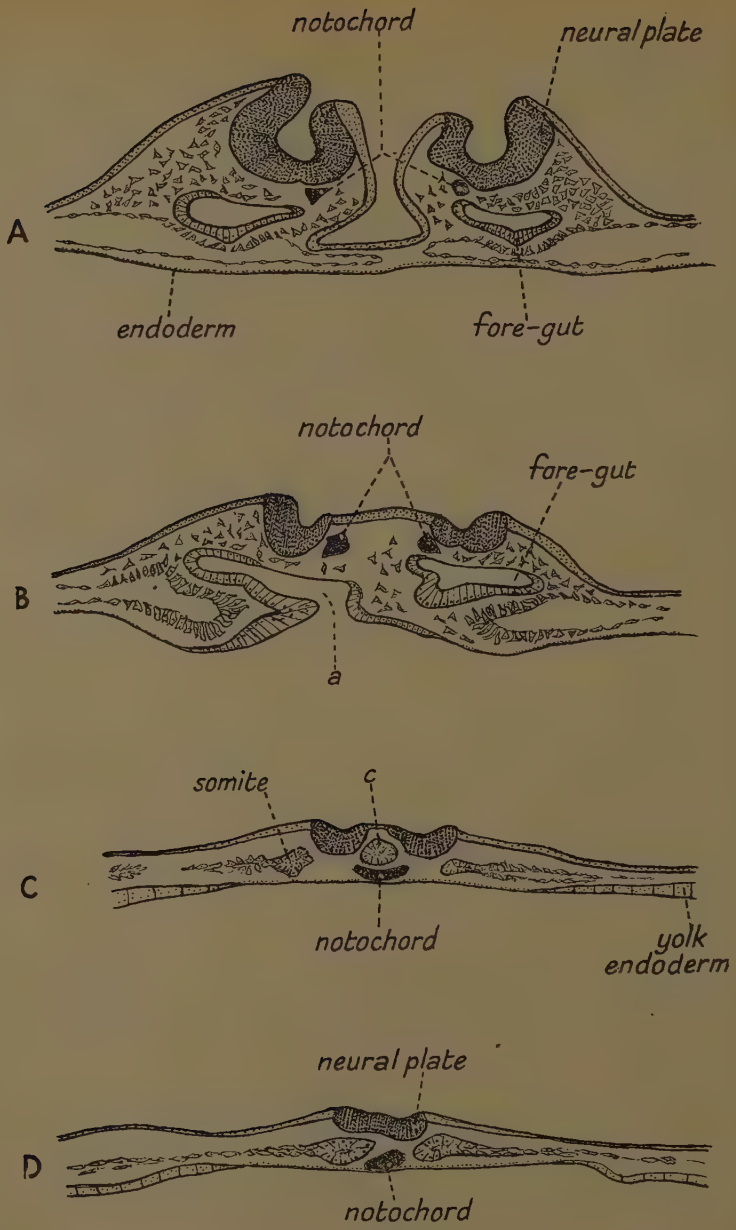
In nine control specimens the node was cut out at L or LM stage, and then replaced in its original orientation. Eight developed into normal embryos, while one failed to heal and formed a split embryo, with notochord along one side of the hole in the axis. These specimens, together with the similar controls reported by Abercrombie (1950), indicate that provided healing occurs mere operative replacement of the node is unlikely seriously to disturb the normal course of development.

Seventy-two embryos in which the node was replaced from the posterior end of the primitive streak were fixed after 24 hours *in vitro*. Those which failed to show any development were discarded before fixation. The fixed specimens fall into two main classes: (1) those with definite axial doubling, and (2) those with an almost entirely single axis. There was no statistically significant difference between the two classes in their age at operation, judged either by the Waddington scale or by the ratios of primitive streak length to area pellucida length.

1. *Embryos with doubled axis*

More than half of the embryos fixed showed signs of axial doubling. Since the notochord is the only midline structure recognizable with certainty, doubling of the axis may be unequivocally said to have occurred only when two notochords are present lying parallel to each other. Sometimes the form and arrangement of two neural plates and of their underlying somites or head mesenchyme may be strong evidence for a real duplication; but at other times it is uncertain whether the appearance of two neural plates is not rather due to the failure of the midline to differentiate. Twenty-eight embryos (39 per cent. of those fixed) have duplications of part or all of the notochord, with or without other signs of doubling. Seventeen embryos (24 per cent.) have the diagnostically less satisfactory duplication of neural plate and mesoderm without duplication of notochord.

(*a*) *Doubled axis with doubled notochord.* As an illustrative example we describe a specimen (No. 8–272) on which operation was performed at L stage, when its primitive streak was 1·5 mm. long (equal to 75 per cent. of the length of the area pelludica). The deleted region of the anterior end of the streak was 200μ long and 300μ wide. The graft was taken from the posterior end of the primitive streak of the same blastoderm. After 24 hours *in vitro* the head and anterior part of the trunk was doubled, while the posterior part of the trunk was single. The heads have very short head-folds and no optic vesicles. There is a



TEXT-FIG. 1. Embryo (No. 8–272) with anterior axial doubling; two notochords are present anteriorly. Magnification ×90. A, anterior head region with closed foreguts. B, head region, at the level of one anterior intestinal portal (*a*). C, trunk region where notochords have fused but neural plates are separate; *c*, central somite. D, posterior part of trunk region with single axis.

long closed foregut (Text-fig. 1A) with a separate heart rudiment in each (Plate, fig. A). The notochords end anteriorly at the normal level; they are strongly displaced towards the original midline of the blastoderm. In the posterior part of the heads the neural plates are small, and the foreguts open by separate anterior intestinal portals (Text-fig. 1B). Between the two axes at this level there is some central mesoderm which as the trunk region is reached becomes a central somite. The two notochords fuse beneath the central somite in the anterior region of the trunk, forming a median notochord which is crescent-shaped in section (Text-fig. 1C). Farther posteriorly the two neural plates fuse, the notochord is displaced to one side of the central somitic mesoderm which becomes continuous with one of the lateral somite series, and an asymmetrical but single posterior end of the trunk region follows (Text-fig. 1D).

We now consider the main generalizations that can be made about the structure of the specimens with doubled notochord.

The two axes in all the doubled embryos converge posteriorly. In 21 specimens the anterior part of the differentiated embryonic axis is, in some or all of its components, doubled, and the posterior part is single. The relative extent of these two parts varies greatly, and the variation cannot be correlated with variation in the size of the graft. In the 7 remaining specimens no part of the differentiated axis is completely single, though the two axes join a single primitive streak. Measurements in sections of the extent of the doubled and single regions were made on 10 specimens picked at random. The anterior doubled region varied in length from 0·6 to 1·6 mm. (mean 0·90 mm.); the posterior single region varied in length from 0 to 0·6 mm. (mean 0·27 mm.). The doubled region tends therefore to be considerably longer than the original graft (which averaged 0·2 mm.). Part of this doubling is prechordal, but since a length of 0·3 to 0·9 mm. (mean 0·53 mm.) of the notochord-containing region of the axes was doubled, and since the anterior tip of the notochord tends to be situated abnormally far posteriorly in these doubled embryos, it may safely be concluded that the doubling usually extends far behind the original position of the node as well as in front of it.

The prechordal part of the head in these specimens always has two separated condensations of mesenchyme and two separated neural tubes or plates. It is probable, however, that the prechordal doubling is defective, since no optic vesicles have been identified. In each of the specimens, except 3 with very distorted heads, there are two closed foreguts; but usually only one, or neither, of the pair of heads has a head-fold.

In 7 specimens the two axes are *completely* separate from each other (i.e. epidermis separates the neural plates, and mesoderm of lateral plate character separates the axial mesoderm) only in the prechordal head, the doubling farther back being only partial. In the other specimens complete separation extends a variable distance back through head and trunk; in 2 specimens right back to

the primitive streak. Commonly there is some rather disorderly mesoderm between the separate axes, probably derived from the graft. In 4 specimens this mesoderm forms somites in the trunk region, where the two axes are close together, so that the central somitic mesoderm is continuous with the medial row of somites of each of the axes.

The two separate axes are often strongly asymmetrical, being deficient on the medial side. The asymmetry is most obvious in the medial displacement of the notochord relative to the neural plate, which may be very extreme as in specimen No. 8–272 already described. The asymmetry is usually, but not invariably, maximal at or near the anterior end of the axis. Even when the asymmetry is most extreme the notochord is bordered medially by some axial mesoderm.

Behind the region where the axes are completely separate, in most specimens, the two neural plates fuse to form a normally structured single neural plate, though the two notochords remain apart. In 4 specimens, however, including the one described above, the notochords fuse with each other anteriorly to the junction of the neural plates. Between the two notochords in the region with a single neural plate there is almost always mesoderm in the midline. When it lies in the head this mesoderm is partly typical head mesenchyme, though sometimes it forms rather denser masses; in the trunk it usually forms a single central somite which is often less well differentiated than the lateral somites and may be associated with extra unorganized mesoderm. It does not form side-plate mesoderm.

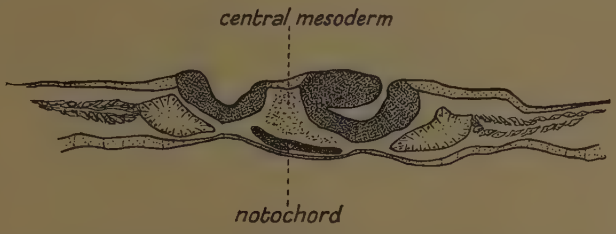
There are abnormalities in the longitudinal extent of the notochords in many of the doubled specimens. In 17 specimens the anterior limit of one notochord is well in front of that of the other. The more anterior one arises at approximately the normal level in 8 specimens; well behind it in the others. The more posterior one may not start till the anterior end of the trunk. Even when the two notochords both have the same anterior limit (11 specimens) this is at the normal level in only half of the specimens; in the others it is more posterior.

Although it is so common for the notochords to be absent in the most anterior part of the axis, they are usually otherwise complete in their longitudinal extent. In 3 specimens, however, one or both notochords end before the posterior limit of the differentiated axis is reached; in two of these the place of the missing notochord is taken by somitic mesoderm.

In 5 specimens the shape in transverse section of the notochord is a dorsoventrally flattened ovoid sometimes as much as 8 times wider than it is deep (Text-fig. 2). This shape persists through many sections, and it is not therefore simply due to the plane of section of the paired notochords at their junction.

Three specimens in which the graft had been marked with phosphorous[32] yielded typical embryos with doubled notochords. In these, complete doubling of all axial components only occurred in the prechordal region of the head, where graft tissue was absent. In no specimen was there any sign that any part of the notochords was graft-derived. The graft was mainly found contributing

to the central mesoderm between the notochords in the head and anterior part of the trunk where the neural plate was single (Plate, fig. B). Farther posteriorly the central mesoderm was of mixed host and graft derivation and farther back still of exclusively host derivation. In no specimen was there graft tissue caudal to the point where the notochords fused. The anterior limit of the graft varied a little. In one it was anterior to, in one about level with, and in one behind the

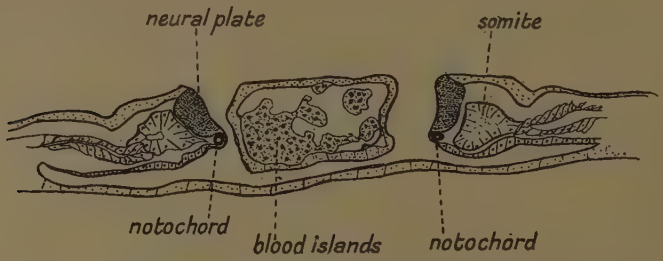


TEXT-FIG. 2. Embryo with doubled axis. The two notochords have joined medially, forming a central very wide and flattened notochord. The central mesoderm is not arranged as a somite. Magnification ×90.

most cranial point reached by either notochord. All three grafts were drawn out lengthwise. Their length at operation was 190μ, their lengths after fixation, 420μ, 500μ, and 540μ. No graft tissue occurred in the overlying neural plate; but the midline endoderm below the graft mesoderm was usually graft-derived.

It is of interest to determine how in these doubled specimens the elimination of the original node has affected the amount of axial material present in the embryo at fixation. In 10 randomly chosen embryos the volume of neural and notochordal tissues was compared with that in 10 single embryos, all of which had undergone some operation (control replacement or antero-posterior reversal) on the node. The volumes of tissue were estimated by measuring with a planimeter the areas in drawings of every tenth section made with a projection microscope. The total volume of neural tissue obtained by multiplying the measured areas by the thickness of 10 sections was slightly larger in the single than in the double embryos (mean and standard error $1{,}650 \pm 210$ against $1{,}520 \pm 280 \times 10^4$ cubic microns). The neural plate of the single embryos tended to be slightly longer than those of the double ones ($1{\cdot}44 \pm 0{\cdot}19$ mm. against $1{\cdot}23 \pm 0{\cdot}12$ mm.), so that the mean cross-sectional area of the neural tissue of the sampled sections was practically identical in the two groups of embryos ($11{,}600 \pm 1{,}500$ square microns in the single ones, $11{,}700 \pm 1{,}400$ square microns in the double ones). The mean total volume of notochord tissue was $55 \pm 9 \times 10^4$ cubic microns in the single embryos, $63 \pm 12 \times 10^4$ cubic microns in the double ones. Again, the total length of the chordal part of the axis was a little shorter in the double embryos ($0{\cdot}78 \pm 0{\cdot}10$ mm.) than in the single embryos ($1{\cdot}00 \pm 0{\cdot}10$ mm.). The mean cross-sectional area of notochordal tissue was 600 ± 120 square

microns in the single embryos, 770 ± 110 square microns as the sum for the two notochords in the double embryos. None of these differences between double and single embryos in neural or notochordal tissue can be considered significant. Analysis of covariance, applied to tissue volumes so as to eliminate the effect of the regression of volume on length of embryo, also failed to show any significant difference between single and double embryos. It seems therefore highly probable that in these doubled embryos the mass of presumptive notochord and neural plate eliminated with the node has been effectively replaced; but that this replacement does not go so far as the development of two axes of normal size. The total amount of neural and notochordal tissue is approximately the same in a blastoderm whether it carries a single or a doubled axis.



TEXT-FIG. 3. Split axis with notochord at each side. The free central graft contains blood-islands. Magnification ×90.

Four rather aberrant specimens remain to be mentioned. One specimen has triple notochords. There are two notochords as usual throughout the length of the axis (one starting rather posteriorly) separated by a central strand of mesoderm which is somitic in the trunk in the usual way. For a short stretch, however, there is an extra notochord lateral to one of the full-length notochords, separated from it by somitic mesoderm except for a brief space where these two notochords fuse. The extra notochord disappears posteriorly without merging with its neighbouring notochord. Another 3 specimens with doubled notochord are typical representatives of the longitudinally split mode of development described below, except that there is a notochord on each side next to the split throughout most of its length (Text-fig. 3). The two notochords join behind the split to form a single axis. The graft (blood-islands and mesenchyme) could be identified in one specimen, lying free in the split.

(*b*) *Doubled axis, notochord not doubled.* There are 17 specimens of this kind, in which the disposition of neural plate and head mesoderm or somites indicated that the axis was doubled. We exclude embryos the doubling of which is confined to the tip of the prechordal head, since this anomaly occurs occasionally after damage to the node produced by a variety of other operations. In 12 specimens the notochord was single throughout, or practically so, and in

5 there was no notochord. Apart from their notochords these specimens are much like those already described, the two axes converging posteriorly, with a varying extent of posterior fusion. Some specimens differ from double-notochord ones merely in that there is a notochord in only one of the pair of axes (Text-fig. 4), and at some point behind the junction of the two axes this notochord takes up a central position. In 2 specimens there are very brief lengths of a second notochord in the second axis. Other specimens differ in that notochord

TEXT-FIG. 4. Embryo with doubled axis. Only one axis has a notochord; the other has somitic mesoderm in place of notochord. The central mesoderm between the two axes is unorganized at this level. Magnification ×90.

is absent throughout the whole doubled anterior region, and only appears, in a central position, in the more posterior single part of the axis; an arrangement transitional to that of certain twins with doubled notochord where both notochords start far back in the axis. In almost all specimens where the notochord of an axis is absent head mesoderm or somite is present in the expected position of the notochord.

The position of fusion of the two neural plates varies as in the double-notochord twins, and in 2 specimens the axes are doubled right back to the primitive streak. In 1 specimen the notochord stops before the node and is replaced by somitic mesoderm with which it is continuous.

On the whole these specimens are not as well differentiated as are those with doubled notochord. Where one axis has a notochord and the other not, the former axis is usually the larger in transverse section (cf. Waterman, 1936).

Where the two axes are separate the mesoderm between them, which is probably usually graft derived, is commonly structureless, and cannot be assigned to any type of tissue. In 3 specimens, however, it contained traces of side-plate mesoderm, and in 3 others traces of a somitic arrangement. In specimens where one of the two axes has a notochord while the other has not, there is a zone where the two axes have come together but have not completely merged. The central mesoderm here, as in the corresponding situation in double-notochord twins, commonly has a somitic structure. It was shown to be graft-derived in 1 specimen marked with phosphorus^{32}.

In 3 specimens the dorso-ventrally flattened notochordal form already

described appears where the anteriorly unilateral notochord takes up a central position in the posterior single part of the axis.

(*c*) *Early fixation.* Five specimens were fixed after 6 hours in culture in order, if possible, to detect early stages in the development of axial doubling. These specimens reached the equivalent of a head-process stage with the neural plate differentiating but not the mesoderm. The graft was to some extent identifiable, because in places it had not entirely merged with the surrounding mesoderm. The graft seemed to have extended lengthwise and to lie now below the surface, occupying the position of the head-process and covered by thickened ectoderm. In 1 specimen something like a node seemed to have re-formed overlying the posterior part of the graft. Behind the graft in all specimens lay the primitive streak. No sign of a lateral node-like structure appeared in any of these specimens, in a position where it might be forming one of the expected pair of axes; but in 2 specimens there were distinct signs of thickening of the blastoderm parallel to, but some distance lateral to the graft and to the anterior end of the primitive streak. The thickenings were mainly in the neuralizing ectoderm, but partly also in the underlying mesoderm. They may well have been incipient double axes, which would later have moved closer together with the concentration towards the midline which accompanies axial differentiation. Forward extension of the head material had not occurred. The axis in front of the graft was apparently always single, with mesoderm and neural plate normally arranged.

2. *Embryos with single axis*

The 27 embryos with a single axis fall into three categories.

(*a*) *Single axis without notochord.* There are 7 such specimens. Two are grossly distorted. The other 5 all have an interesting feature: they have side-plate mesoderm in the midline of the axis, where the notochord should be (Plate, fig. C), at least in the anterior part of the axis. The neural tissue, in spite of the abnormality of the underlying mesoderm, seems qualitatively within the normal range though quantitatively somewhat inferior to it. The somites do not appear to be reduced in size, except in the anterior part of the trunk in 1 specimen, where instead of somitic tissue there is only thickened side-plate (Plate, fig. D). Posteriorly, the region of midline side-plate is succeeded by midline mesoderm of somitic structure in 3 specimens. In one of these 3 specimens the midline somitic mesoderm is not continued far back; it is succeeded by two brief patches of notochord, then simply by a midline gap between the well-developed somites. In another of these specimens the phosphorus^{32} marking technique had been used, and the midline side-plate and the succeeding midline somite-like mesoderm both proved to be graft derived, the rest of the axis being formed from the host. Two of the five specimens with midline side-plate have doubling of the extreme anterior of the prechordal part of the head.

(*b*) *Single axis, longitudinally split.* In 11 specimens, through failure of heal-

ing, the axis is split along a varying part of its length. Such specimens are well known in chick experimental work and have been discussed most recently by Abercrombie (1950). The present group of specimens is in one respect different from those obtained by Abercrombie (1950). In 7 of the present group, in all of which only the node, i.e. less than 0·3 mm. of the anterior end of the streak was removed, the notochord was totally absent. In those previously obtained by reversed transplantations involving a similar amount of the streak, notochord was totally absent in only 1 out of 10 specimens; in the other 9 it was found in the part of the axis behind the split. In 1 of the present 7 specimens there is midline somitic mesoderm instead of notochord in the unsplit posterior part of the axis. Besides these 7 specimens with no notochord, there were 2 with posterior notochord. The remaining 2 were unlike previously described specimens in that they had a notochord along one side of the split, in one case throughout the whole extent of the expected chordal region of the embryo, in one other in two separated patches, one in the head and one in the trunk. These specimens lead on to the specimens already described in which there was a notochord on both sides of the split.

In these split specimens the head is commonly extremely distorted, broad and short and split to near its anterior limit. Presumably the head material has here failed to extend forwards in the usual way. In 3 specimens a single median foregut and heart extends a little forward of the main mass of the head; while in 3 others (including the 2 with unilateral notochord) there is definite doubling of the most anterior part of the head.

(*c*) *Single axis with notochord.* Nine specimens come within this category. Four are within the range of normality found in embryos grown in tissue culture, while 5 have minor abnormalities—chiefly extra disorderly mesoderm in the axis (together with an extra somite in one specimen) or a slight doubling of the neural groove within a single continuous neural plate. One of them has a brief stretch of duplicated notochord about 50μ long in the trunk region.

DISCUSSION

The most interesting result of operations in which the primitive node is replaced by the posterior part of the streak in blastoderms of stage LM or L is the high proportion of doubled embryos which develop. Bautzmann (1933) by analogous operations in amphibia has obtained duplications, though of a much less extreme degree. He found that when he removed from an early gastrula a median piece of the dorsal marginal zone (i.e. the median part of the presumptive notochord, not as in the chick the whole presumptive notochord), and replaced it by a piece from the lateral or ventral marginal zone, he obtained embryos with anterior doubling, in particular of the notochord. The similarities and differences between the chick and amphibian results will be discussed when we describe experiments in a future paper bearing on the causal relations involved in the initial duplication of the axis-forming system. At present we wish

merely to discuss the disposition of structures in the doubled embryos and its probable course of development. The surest criterion of axial doubling is the presence of doubled notochord, and we will discuss first the duplication of this structure.

The notochord is laid down in a normal embryo as a result of the backward extension of the primitive node, within which is concentrated the presumptive area of this tissue (Wetzel, 1929; Pasteels, 1937). That a backward movement occurs in our double embryos is indicated by the considerable longitudinal stretching which the grafts marked by phosphorus^{32} have undergone. It is reasonable, therefore, to suppose that in effect two primitive nodes have functioned. We assume that as a result of the operation the deleted node was functionally replaced by two regions with nodal properties, situated side by side, which then laid down two approximately parallel notochords. Although they functioned like normal nodes, it was not possible to identify the characteristic structure of a node in these regions of presumed new nodal properties when operated blastoderms were fixed 6 hours after the operation.

The next problem is to decide what material went to form the two regions of nodal properties. The graft itself contributed nothing to the notochords, judging by the experiments where phosphorus^{32} marking was used. Hence it is probable that they developed from the mesodermal material which normally lies at each side of the anterior end of the streak. This material (that is, the mesenchyme which was observed at either side of the defect in blastoderms fixed immediately after operation) is of uncertain presumptive fate in normal development, but may be largely parachordal head mesenchyme or somites. Even if fragments of the presumptive notochord remain at either side of the graft, it is clear that their simple self-differentiation cannot account for the notochords found at fixation, since the total volume of differentiated notochord in a doubled embryo is no less than that in a single embryo. It seems likely, therefore, that the operation releases notochordal potency in material which is not presumptive notochord at either side of the graft. Notochordal potency has occasionally been found here (Grobstein & Zwilling, 1953).

This hypothesis of two new nodal regions does not explain the posterior fusion of the doubled notochords which is so commonly found. Our data do not justify us in going beyond the statement of two alternative hypotheses about this. (*a*) The new nodal regions are initially separated by the graft. When the movement of extension occurs, the graft, as well as the new nodal regions, becomes drawn out, but not as much as the nodal regions, so that posteriorly it ceases to be interposed between these. When no longer separated the two node systems fuse, as regulatory systems in general tend to do when in close contact. (*b*) The single posterior part of the notochord develops from the region of the host primitive streak immediately behind the graft, that is, immediately behind the original primitive node. After the operation the graft in a prospective twin may in fact be bordered posteriorly as well as laterally by material which will form noto-

chord. There is no doubt that the primitive streak region behind the graft has notochordal potency (Spratt & Condon, 1947; Abercrombie, 1950); and it may actually be presumptive notochord in a normal embryo. It is curious that there is very great variation from specimen to specimen in the extent of the posterior region with single notochord. This variation seems rather more extensive than can easily be accounted for by variability between blastoderms in their presumptive areas or by variation in operative technique. Phosphorus^{32} marking gives no support to the hypothesis that a varying amount of the posterior part of the graft becomes notochord.

We turn now to consider the disposition of the parachordal mesoderm, that is the somites and head mesenchyme, in doubled embryos. Such mesoderm is on the whole organized around the notochords in typical fashion, though commonly some degree of asymmetry indicates a lack of perfect co-ordination. Doubling of the parachordal mesoderm might be regarded simply as a consequence of the development of two regions with nodal properties, the two differentiating notochords organizing the surrounding mesoderm by a process analogous to the 'Yamada-effect' in amphibia (Bautzmann, 1933; Yamada, 1940). But amongst our specimens occur doubled axes, one or both of which lack notochord, wholly or partly, the missing notochord being replaced by parachordal mesoderm. Notochord differentiation is therefore unnecessary for the initial formation of parachordal mesoderm in laterally displaced axes. It is also known to be unnecessary for the initial formation of parachordal mesoderm in its normal position in a single axis (contrary to the opinion expressed by Spratt, 1942): parachordal mesoderm frequently develops after elimination of the notochord (e.g. Waddington, 1935; Waterman, 1936; Wetzel, 1936; Wolff, 1936). In the present experiments such differentiation also occurred in those specimens with a single axis the notochord of which is replaced by a strand of central sideplate mesoderm. Doubling is therefore a more complex matter than the mere formation of new nodes, but the interrelations within the chorda-mesoderm are not sufficiently clear to warrant further discussion.

The prechordal part of the axes may conveniently be discussed as a unit. This region normally forms as a result of a forward movement of presumptive mesoderm and neural plate, occurring perhaps rather later than the backward movement which forms most of the chordal region of the axis. Both these movements are probably to be regarded as two aspects of the longitudinal extension and lateral narrowing of the axial material which follows the substantial completion of invagination.

The prechordal head is almost invariably doubled (or at least separated into two lateral parts) when there is any degree of doubling in the rest of the axis. The only exceptions are a few specimens in which evidently no forward movement has occurred and the head is grossly distorted; and Abercrombie (1950) described a specimen with normal single prechordal head but with the notochord doubled, the two notochords being exceptionally close together. Doubling

of the prechordal head is not, however, confined to specimens with a doubled axis. It occurs, though infrequently, after operations on the node which have not produced doubling of any other part, for instance in otherwise single specimens in which the notochord is replaced by side-plate or somitic mesoderm, or in split specimens.

Doubling of the prechordal head seems always to be a very imperfect process, more so than the doubling of the chordal part of the axis. The two heads lack any trace of optic vesicles, though this may be partly explicable by delayed development (cf. Wolff, 1948). The disposition of neural plate, head mesoderm, and foregut shows, however, that we are dealing with more than the mere independent development of the lateral parts of the original presumptive prechordal region.

The midline material of the presumptive prechordal head, which lies in front of the operation site, must have failed to move forward in all of these doubled specimens. In some cases we can go farther and say that it has not differentiated *in situ* either, because if it had done so the two axes would be linked by central neural plate and mesoderm somewhere in the head region; yet this is not so in those specimens where the axes are completely separate back to the trunk region. The problem of what becomes of this midline presumptive prechordal mesoderm is made particularly acute by the numerous indications that this material is relatively strongly determined at primitive streak stages. When isolated at L stage, for instance, and grown as a chorioallantoic graft the material anterior to the streak often forms fore-brain (Stein, 1933; Dalton, 1935). The same tendency has been shown in tissue culture (Waterman, 1936; Rudnick, 1938) and after destruction of the node *in situ* (Dantchakoff, 1932; Wetzel, 1936). Yet when doubling of the axis occurs this pre-nodal material apparently fails to differentiate. Perhaps, however, there is no real failure of differentiation, but an anomaly of tissue movement. It seems that in the doubled specimens the forward movement of prechordal material tends to occur particularly in front of the midline of each of the two new chorda-mesoderm systems: and it is possible that these bilateral forward movements draw into themselves the original midline material, which is thus removed from the scene of its normal differentiation and contributes to each of the two heads.

Little need be said about the arrangement of the rest of the neural plate in double embryos. It would be expected to follow the disposition of inducing tissues (notochord and parachordal mesoderm), and on the whole it does. When there are two widely separate chorda-mesoderm axes there are two neural plates, each bilaterally symmetrical. When the chorda-mesoderm, though doubled, is not divided by a midline strip of non-inducing mesoderm, there is a single bilaterally symmetrically neural plate. In the latter case, judging by the specimens marked with phosphorus^{32}, the neural plate is not graft-derived.

The two axis-forming systems which produce a doubled embryo are highly correlated with each other in their development. The invariable identity of stage

of development at each level of the two axes shows that the backward progression of their differentiation must have started at the same time and proceeded at the same rate; the two axes show close correspondence of antero-posterior levels (except for the anterior tip of the notochord); and they always have an almost parallel, slightly convergent, orientation. The correlation may be due to their position in the middle of a common area pellucida, their mode of origin from a single initial axis-forming system, or their proximity to each other. The two axes, of course, are often sufficiently near each other to share to a considerable extent common medial parachordal mesoderm (graft-derived). Similar correlated development is shown when a grafted primitive streak develops an axis close to that of the host (Waddington & Schmidt, 1933; Abercrombie & Waddington, 1937).

The question as to how the axis-forming system comes to be doubled as a result of the operation we have performed is not simple, and its consideration will be deferred to a subsequent paper, as will the relation of our specimens to the doubled axes obtained experimentally by others, particularly by Wolff (1948) and Lutz (1949), and to those which sometimes occur naturally. It remains to discuss the single axes which we sometimes obtained.

The most interesting type of single embryo amongst our specimens is that with median side-plate mesoderm in place of the notochord. Embryos of such structure have not to our knowledge been described before. The development of the graft is here distinctly different from its development in doubled embryos. In the latter, where the graft has been positively identified (in phosphorus^{32} marked embryos) it has formed, apart from endoderm, medial somite or head mesenchyme. Unfortunately it is not known what the graft forms in those doubled specimens where the axes are widely apart. The graft in these seems to be very thoroughly incorporated into the blastoderm between the two axes and/or into the two axes themselves. In the specimens with single axis under discussion the graft has apparently formed median side-plate and has not developed simply according to its presumptive fate, which included blood-island. Under the influence of its position it has formed the thickened side-plate which normally lies next to the somites. In their posterior parts these grafts, which become elongated during development, tend to form somitic mesoderm, a development which is possibly due to the greater time available for modification before differentiation begins in the posterior part of the axis (cf. Bautzmann, 1933; the same time factor in differentiation might also be invoked to explain the failure of the notochord to develop in the anterior parts of many double specimens).

The most surprising feature of these embryos is that the neural plate (which is probably host-derived) is histologically so normal (though small in section) even in the extreme cases where somites are missing as well as notochord. How has it been induced? Waddington (1936) found experimentally in Urodeles that side-plate mesoderm could induce competent ectoderm, so there is good precedent for the view that the neural plate has been induced by the material which

is found underlying it. An alternative view, that the neural plate has moved into position after induction by more anteriorly or laterally situated presumptive axial material, has, however, become a possibility as a result of the mapping of presumptive neural plate by Spratt (1952). Little is really known of the time and place of normal neural induction in the chick.

In some single embryos the place of the notochord is partly taken by graft-derived somitic mesoderm. The next and last step in the conversion of the posterior graft along the scale of mesoderm differentiation would be for the graft to develop into notochord, resulting in a single almost normal axis, and a few such embryos occurred.

SUMMARY

1. In chick embryos with a full-length primitive streak the primitive node has been replaced by a graft from the posterior end of the primitive streak, and the blastoderm subsequently cultivated by Waddington's technique for 24 hours.

2. More than half of those embryos which developed at all showed a doubling of the anterior part of the axis, variable in extent but considerably exceeding the original extent of the graft, with convergence to a single posterior part. Prechordal head, parachordal mesoderm, and neural plate were affected by the doubling. Most specimens had two notochords fusing posteriorly into a single one, or occasionally terminating independently at a common primitive streak. Other specimens had a notochord in only one of the two axes, or in neither.

3. Marking experiments with phosphorus^{32} on a few specimens showed that the graft, which had become greatly elongated, contributed the mesoderm between the notochords, but did not form any notochordal tissue.

4. The doubled axes were often asymmetrical, and in the head region probably incomplete. The total amount of neural and notochordal tissue in a doubled embryo was not significantly different in volume from that in a single embryo.

5. In a few specimens in which the axis remained single the place of the notochord was taken by side-plate mesoderm, probably graft-derived. Posteriorly this midline mesoderm became somitic. The neural plate in such specimens was little if at all reduced.

6. Other specimens failed to heal and developed a split axis. They were usually devoid of notochord, but occasionally had a notochord along one side of the split, or along both sides.

7. Finally, a few specimens formed an almost normal single axis.

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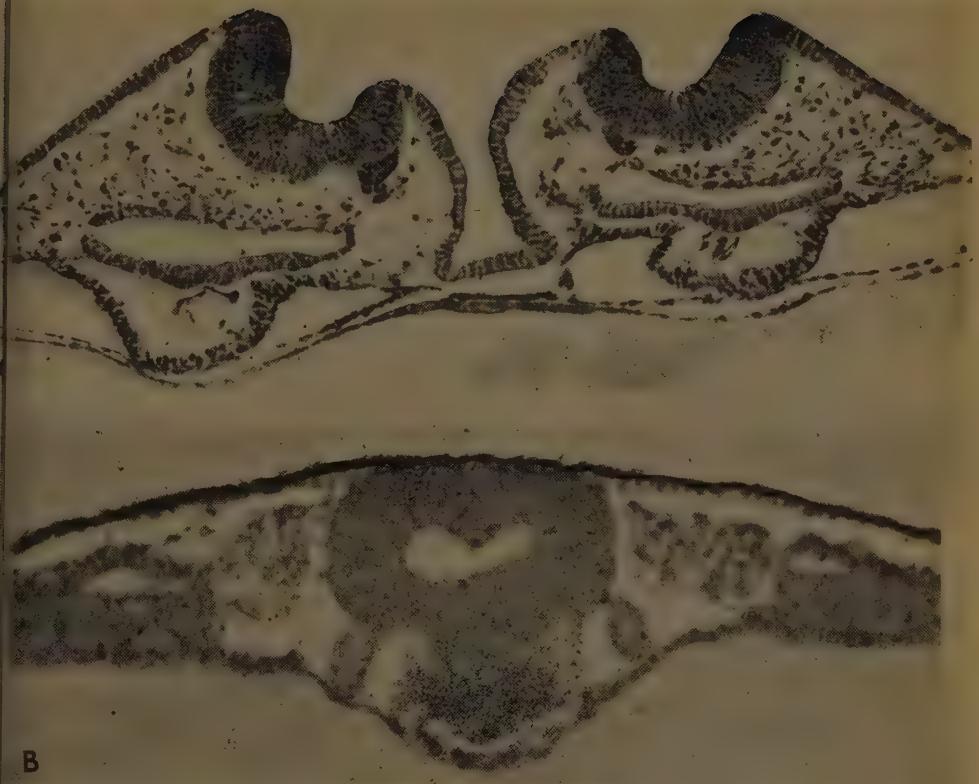
EXPLANATION OF PLATE

FIG. A. Head region of an embryo (No. 8–272) with doubled axis. Two neural plates, foreguts, heart rudiments and medially displaced notochords. Magnification ×200. (Previously reproduced in Waddington, 1952.)

FIG. B. Autoradiograph of an embryo with partially doubled axis. The (somitic) mesoderm between the two notochords is covered with silver granules, indicating derivation from the phosphorus^{32}-marked graft. The blackening of the epidermis is an artefact. Magnification ×270.

FIG. C. Trunk region of an embryo with single axis, the notochord of which is replaced by side-plate mesoderm. Magnification ×200.

FIG. D. Trunk region of embryo with single axis, both notochord and somites being replaced by side-plate mesoderm. Magnification ×200.



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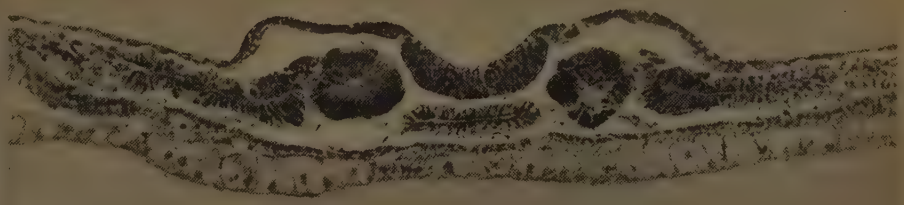

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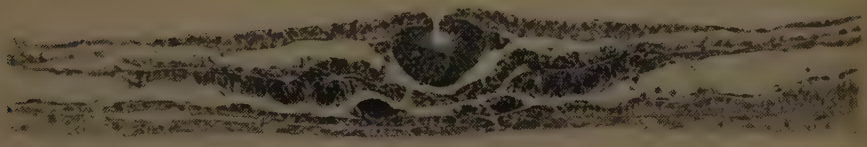

D

Transplantation of Living Nuclei of Late Gastrulae into Enucleated Eggs of *Rana pipiens*

by THOMAS J. KING *and* ROBERT BRIGGS[1]

WITH ONE PLATE

In previous papers it has been shown that living nuclei of animal hemisphere cells of frog blastulae and early gastrulae can be transferred into enucleated eggs (Briggs & King, 1952, 1953*a*). Approximately 20 per cent. to 40 per cent. of the recipient eggs cleave normally, and the majority of these develop into complete embryos, demonstrating that the transplanted nuclei are undifferentiated (i.e. still capable of participating in all types of differentiation). This result is more or less expected since the nuclei in question come from undifferentiated parts of the blastula and early gastrula.

The next step in this analysis was to test the nuclei from areas of the late gastrula which are regionally determined, namely, the chorda-mesoderm and the presumptive medullary plate. Embryological evidence indicates that the chorda-mesoderm is determined to differentiate into mesodermal structures, while the overlying presumptive medullary plate has at least a labile determination in the direction of neural differentiation. However, it is not known whether these regional determinations involve stabilized differentiation of the constituent cells. This question will be discussed after we have presented the results of experiments in which nuclei from chorda-mesoderm and presumptive medullary plate were transferred into enucleated eggs.

The areas from which nuclei were taken for transplantation are indicated in Text-fig. 1. Donor cells were obtained from the median plane, in a region approximately midway along the longitudinal axis of the embryo. In the case of the chorda we used only cells from the two uppermost layers, i.e. the layers nearest the overlying presumptive medullary plate. Presumptive neural nuclei were provided by the two lower cell-layers of the presumptive medullary plate, these being the layers adjacent to the chorda. In all cases the nuclei were transplanted by means of the technique previously described (Briggs & King, 1952, 1953 *a*, *b*). The procedure involves first enucleating the recipient egg, and then transferring into it a single nucleus from a donor cell. The nucleus must be protected by its own cytoplasm in the course of the transfer, which means that donor

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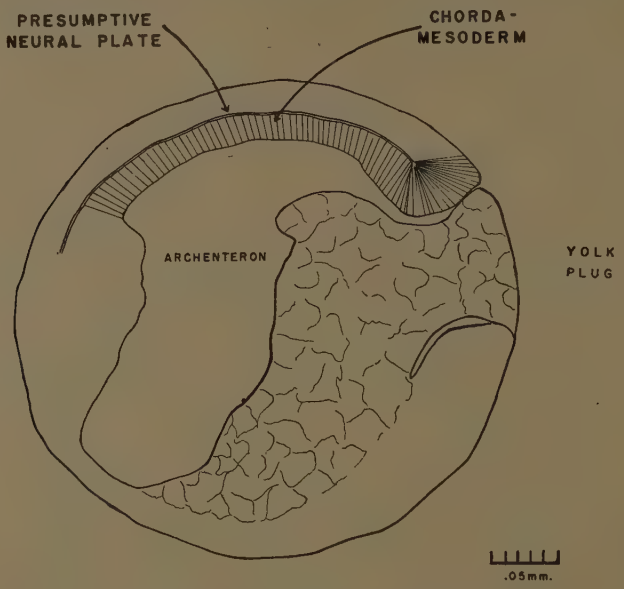
cell cytoplasm is introduced along with the nucleus into the recipient egg. Quantitatively the cytoplasm thus included is insignificant, being only about 1/600,000 the volume of the recipient egg. None the less, it must be considered



TEXT-FIG. 1. Camera lucida drawing of a frog gastrula at the yolk plug stage (stage 12 of Shumway, 1940). The arrows indicate the areas from which donor nuclei were taken.

in the interpretation of results, as we shall indicate below. Another important technical matter has to do with the fact that the operation is not perfect, and under the best circumstances results in damage to some of the transferred nuclei (see Briggs & King, 1953*a*). This must also be considered in appraising the significance of the results to be presented.

RESULTS

As we have reported previously (Briggs & King, 1952), enucleated eggs which are left untreated or are injected with cytoplasmic granules never display cleavage. When these eggs are injected with nuclei from late gastrulae the majority do show cleavage of one sort or another, as indicated in Table 1. In about one-fourth of the total number of cases the cleavage is abortive and fails to give rise to blastulae. The remainder of the cleaved eggs develop into partial or complete blastulae. Some of the partial blastulae are made up largely of cells lacking nuclei and resemble the 'nucleusless' blastulae produced by inseminating enu-

cleated eggs with heavily irradiated sperm (Briggs, Green, & King, 1951). The remainder of the partial blastulae are normal in appearance except for the presence of an uncleaved area. This area is quite variable in size, ranging from less than one-tenth to roughly one-half of the blastula surface. Finally, the complete blastulae, comprising 8–9 per cent. of the total number of eggs, are of normal appearance. As will be apparent from the table, there is no significant difference in the behaviour of eggs containing transplanted chorda-mesoderm nuclei and those containing nuclei from the presumptive medullary plate.

TABLE 1

Cleavage of enucleated eggs injected with chorda-mesoderm and presumptive medullary plate nuclei

				Blastulae		
				Partial		
Source of nuclei	*Total eggs injected*	*Uncleaved*	*Abortive cleavage*	*'Non-nucleated'*	*Nucleated*	*Complete*
Chorda-mesoderm	242 (100%)	80 (33%)	68 (28%)	46 (19%)	28 (12%)	20 (8%)
Pre-medullary plate	271 (100%)	102 (38%)	76 (28%)	57 (21%)	11 (4%)	25 (9%)

From these results we can say that at least some of the nuclei from determined areas of the late gastrula are capable of participating normally in cleavage and blastula formation when transferred back into enucleated eggs. However, a larger number of nuclear transfers result in abnormal cleavage, partial cleavage, or in no cleavage at all. While we cannot exclude the possibility that some of these failures may be due to intrinsic properties of gastrula nuclei rendering them incapable of entering normally into the cleavage cycle of the egg, the evidence indicates that the majority of the failures actually result from damage inflicted on the nuclei in the course of the transfer. A detailed study, including cytological evidence of damage to the transplanted nuclei, has already been published (Briggs & King, 1953*a*). Here we should like only to point out that the main hazard in the transfer procedure is the inadvertent exposure of the nuclei to the medium in which the operation is carried out. With the relatively large cells of late blastulae the cytoplasmic protection afforded the nuclei during the transfer is such that about one-third of the attempted transfers are completely successful, leading to normal cleavage of the recipient eggs. With the smaller cells of early gastrulae, and the still smaller ones of late gastrulae, the amount of cytoplasm protecting the nucleus is diminished and the proportion of the attempted transfers which turn out to be successful is correspondingly reduced (see Table 2). This evidence, along with the cytological work referred to above, indicates that the majority if not all of the failures in cleavage are due to nuclear damage, and not to changes in the properties of the nuclei during development.

Having established the fact that chorda-mesoderm and pre-medullary plate nuclei can participate normally in cleavage, the next step in the investigation was to find out how they might function during later stages of development. For this purpose we followed the development of the eggs which cleaved normally

TABLE 2

Transplantability of nuclei of blastulae and gastrulae

Stage	*Age-hrs. at 18°*	*Donor cell type*	*Approx. vol. of donor cell* $\times 10^{-5}$ *cu. mm.*	*Total No. recipient enucleated eggs*	*Complete blastulae obtained*	
					No.	*%*
Blastula st. 8–9	18–21	Animal hemisphere	8	204	69	(34)
Early gastrula st. 10	24–28	„	4	135	29	(21)*
Late gastrula st. 12	*c.* 42	Chorda-mesoderm	1	242	20	(8)
Late gastrula st. 12	*c.* 42	Pre-medullary plate	1	271	25	(9)

* Recalculation of the data previously reported (Briggs & King, 1953) showed the number of complete blastulae in this group to be 29 instead of 20. Therefore, 21 per cent. is the correct proportion of injected eggs that formed complete blastulae.

Notes to Table 2

Data on the transplantability of blastula and early gastrula nuclei are taken mainly from a previous report (Briggs & King, 1953*a*). They are included here to provide a comparison with the data on the nuclei of the late gastrulae.

Donor cell volumes were determined from measurements of the cell diameters as seen in sections. For the blastulae and early gastrulae the volumes were calculated on the assumption that the cells are spheres. In the case of the chorda-mesoderm and presumptive medullary plate cells of late gastrula, it was assumed that the cells are cylinders.

The nuclear transfers were carried out in the Niu-Twitty salt solution, except for 167 of the transfers of pre-medullary plate nuclei which were done with 0·9 to 1·9 per cent. oxypolygelatin added to various combinations of the Niu-Twitty salts. The results obtained with the oxypolygelatin did not differ significantly from those obtained with the Niu-Twitty mixture alone.

following nuclear transfer. These gave rise to apparently normal complete blastulae, as has already been mentioned. The later development of these blastulae is summarized in Table 3, which lists the results for blastulae containing nuclei from the determined areas of the late gastrula. For comparison, the results for blastulae containing nuclei from an undetermined area of the early gastrula are also given. An inspection of the table and of the notes attached to it shows that there are no differences in the types of embryos derived from the three classes of blastulae. In all cases some of the embryos are arrested in blastula or gastrula stages. Sections were made of some of these and revealed mitotic irregularities and absence of nuclei in some parts of the embryos—abnormalities probably stemming from damage to the nuclei during transfer. The remaining embryos, representing one-half or more of the total number, continue their development

to neurula and post-neurula stages and display differentiation of all three germ-layers and derivatives thereof. Considering the embryos containing chorda-mesoderm or pre-medullary plate nuclei (lines 2 and 3 of Table 3), there are certainly no differences in extent or type of development that can be correlated

TABLE 3

Later development of blastulae derived from gastrula nuclei

Source of nuclei	*Total No. blastulae*	*Development*			
		Blast.	*Gast.*	*Neur.*	*Post-neur.*
Early gastrula (st. 10)					
Presumptive epidermis .	29	5	5	2	17
Late Gastrula (st. 12)					
Chorda-mesoderm . .	20	6	4	3	7
Pre-medullary plate .	25	8	6	2	9

Notes to Table 3

All of the post-neurulae listed developed into motile 8–10 mm. embryos, some of which were normal while others had slight to moderate abnormalities such as microcephaly and retardation of growth. All embryos externally showed evidence of all three germ-layers, e.g. mouth, anus, suckers, nasal pits, eyes, gills, circulating blood-cells, pronephric protuberances, intestine, and tail somites. Three of these embryos, one from each group, developed into free-swimming tadpoles; a fourth, derived from a chorda-mesoderm nucleus, metamorphosed (see Plate).

Sections, made of 3 or 4 embryos of each group, confirmed that differentiation of the various organ systems had occurred. Structures such as brain, spinal cord, notochord, gut, pronephric tubules, heart, blood-vessels, somites, and sense organs were seen in all 11 of the post-neurulae examined.

Among the neurulae, all of which were abnormal, the most common abnormality was a failure of the neural folds to close normally. Nevertheless, cytological observations of 6 of these embryos showed differentiation of neural tubes, notochord, nephrotomes, and primitive gut.

The ploidy of all post-neurula embryos was estimated at tail-bud stage on the basis of epidermal cell size (see Briggs, R., *J. Exp. Zool.* **106**, 237–66, 1947, for study of validity of method). In 4 cases ploidy was also determined on the basis of nuclear size and nucleolar number in the epidermis of tail-tips from young tadpoles. Results: One embryo, derived from an egg injected with a nucleus from an early gastrula, appeared to be a mosaic, containing both haploid and diploid nuclei. Otherwise, all the embryos were either diploid or polyploid (probably tetraploid). Thus 7 out of the 17 embryos resulting from transfers of early gastrula nuclei were polyploid, while among the embryos derived from transfers of late gastrula nuclei 13 out of 16 were polyploid. As pointed out previously (Briggs & King, 1952) polyploids may arise if the nucleus, following transfer, completes a mitosis before the recipient egg is prepared to cleave.

with the different sources of the nuclei. In both cases the most successful embryos develop into larvae and display advanced differentiation of all cell types. Thus, nuclei from the presumptive medullary plate are *not* limited to participation in neural differentiation but may participate as well in mesodermal and endodermal differentiation. The same situation exists with respect to the chorda-mesoderm nuclei, which also participate in all types of differentiation. Embryos containing nuclei from early gastrulae (line 1, Table 3) are less apt to become

arrested or abnormal during gastrulation and neurulation. This is to be expected since these nuclei are less likely to be damaged in transfer than are those of the late gastrulae. Otherwise, this group of embryos does not differ from the other groups listed in the table.

DISCUSSION

The conclusion to be drawn from these experiments is that some, and possibly all, of the nuclei of determined areas of the late gastrula are not themselves determined or differentiated. In other words, they have not undergone irreversible genetic changes limiting them to participation in only one type of differentiation. Since the experiments involved the introduction of cytoplasm along with the nuclei into the test eggs, it also appears that there are no cytoplasmic genetic units directing differentiation in the late gastrula, or that if there are such units they are no longer effective when transferred back into the egg cytoplasm.

Now, how is this result to be fitted in with the embryological evidence concerning the properties of the chorda-mesoderm and the presumptive medullary plate? The chorda-mesoderm of the late gastrula is quite certainly determined in a general way to form mesodermal structures, and will not give rise to neural or other structures normally derived from ectoderm (Holtfreter, 1933). On the other hand, the presumptive medullary plate, following exposure to the chorda-mesoderm, is determined to form neural tissue, although in the late gastrula this determination may still be labile (Marx, 1925; Mangold, 1929; Holtfreter, 1947). While the evidence indicates that the *areas* are thus determined, it does not tell us whether this determination depends primarily upon differentiation of the *individual cells* or is to be regarded instead as a property of the mass as a whole, the individual cells being still undifferentiated or in a labile state of differentiation. Recent evidence obtained by Grobstein (1952) and by Grobstein and Zwilling (1953) indicates that the second of these two possibilities may be the correct one. These investigators, working with cultured explants of chick blastoderm and mouse embryonic shield, find that the extent of differentiation depends upon the degree to which the explant cells are dispersed. For example, large explants will differentiate into neural tissue; but if these explants are divided into eighths or sixteenths the differentiation fails to occur or is poorly expressed. On the basis of this and other evidence it appears that in chick and mouse embryos organ determination occurs while the component cells are still undifferentiated or are in a labile state of differentiation. The same situation may very well exist in the amphibian gastrula. Our results support this view since they indicate that this phase of differentiation does not require specific irreversible changes in nuclear function, nor does it involve the elaboration of cytoplasmic elements which can direct differentiation when transferred back into eggs. Whether irreversible changes in genetic units are involved in cytodifferentiation during later stages of development remains to be worked out.

SUMMARY

1. Living nuclei were transferred from determined areas of late gastrulae, the chorda-mesoderm, and presumptive medullary plate, back into enucleated eggs (*Rana pipiens*). In both cases about 8 to 9 per cent. of the attempted transfers resulted in normal cleavage of the recipient eggs. The rest of the eggs either failed to cleave or cleaved abnormally. The evidence indicates that most, if not all, of these failures were due to damage inflicted on the nucleus during the transfer procedure.

2. The normally cleaved eggs developed into complete blastulae. Some of these were arrested in blastula or gastrula stage, again as a result of nuclear damage in all probability. The remaining embryos, representing about one-half of the normally cleaved eggs, developed to neurula and post-neurula stages. These embryos displayed differentiation of all three germ-layers and their derivatives, regardless of whether their nuclei were derived from chorda-mesoderm or from presumptive medullary plate.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATE

FIG. A. Ventral view of a 1·1-cm. tadpole derived from an enucleated egg injected with a presumptive medullary plate nucleus of a late gastrula. Nuclear size and nucleolar number, as seen in a tail-tip preparation, showed this animal to be 4 N. (Magnification ×6·5.)

FIG. B. Dorsal view of a 7·2-cm. metamorphosing tadpole derived from an enucleated egg injected with a presumptive chorda-mesoderm nucleus of a late gastrula. Nuclear size and nucleolar number, as seen in a tail-tip preparation, showed this animal to be polyploid, probably 4 N. (Magnification ×1·7.)


A
B

Some Observations on Vitally Stained Rabbit Ova with Special Reference to their Albuminous Coat[1]

by P. BACSICH *and* W. J. HAMILTON[2]

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WITH ONE PLATE

INTRODUCTION

THE gradual deposition of a homogeneous, jelly-like covering on the rabbit ovum during its passage down the uterine tube was first observed by Cruikshank in 1797. This observation has been confirmed repeatedly by many later workers (Barry, 1839; Bischoff, 1842; Thomson, 1859; Assheton, 1894; Gregory, 1930; and Pincus, 1936). Barry referred to this covering investing the zona pellucida as 'chorion', some called it the 'pro-chorion', while others (Lenhossek, 1911; Grosser, 1927) simply spoke of a deposit of mucus due to the glandular activity of the tubal epithelium. In the more recent literature (Gregory, 1930; Pincus, 1936) this layer is generally referred to as an 'albuminous' coat, probably because of its slight similarity to the covering of a hen's egg.

Lenhossek (1911) described a scanty but an identical coat on the ovum of the horse and dog. Hamilton & Day (1945) found a thin coat of material on the tubal ovum of the horse similar to that on the rabbit ovum. In monotremes (Hill, 1933; Flynn & Hill, 1939) and in many marsupials (Hartman, 1916; Hill, 1910, 1918; McCrady, 1938) a thick albuminous coat is laid down on the zona pellucida as the egg traverses the oviduct. The investigations of Hammond (1934) on the fertilization of rabbit ova in relation to time of insemination show that the albuminous coat deposited on the tubal ovum plays an important part in preventing fertilization.

While the early terms 'chorion' and 'pro-chorion' expressed a certain mistaken belief in the probable functional significance of this albuminous covering of the rabbit ovum, until recently neither the true chemical nature nor the probable role played in the physiology of the developing egg by this coat has been the

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subject of a detailed study by modern histochemical methods. In fact, only Braden (1952) in his full investigation of the nature of the zona pellucida of the rat and rabbit eggs has made any observations on the composition and staining reactions of the 'albumen' layer of rabbit eggs.

The pioneer researches of Dalcq (1952 *a*, *b*, *c*) and Dalcq & Massart (1952) on the histochemistry of mammalian ova give details of the vital staining reactions of a number of rodent's eggs and blastocysts, but their interest is focused almost exclusively on the cytoplasmic constituents of the ovum, and consequently their findings will not come within the scope of the present paper, which is concerned mainly with the vital staining reactions of the albumen coat of the rabbit ovum.

MATERIAL AND METHODS

Ten animals were used in the experiment. The animals were killed at varying intervals, ranging from 20 hours to 5½ days, after mating. In 7 instances the aim was to obtain tubal ova, while in 3 cases blastocysts from the uterine horns were recovered. The ova and blastocysts were obtained by flushing either the uterine tubes or the uterine horns respectively with Ringer's fluid containing 0·01–0·05 per cent. toluidin blue. In the case of the tubal eggs the Fallopian tubes were straightened by dissecting away the mesosalpinx and the tubes were flushed from the uterine end towards the fimbriae. To obtain the uterine ova the horns were separated at the cervical junction and were flushed by inserting a cannula at the tubal end. The washings were collected in watchglasses. The ova and blastocysts either appeared in the washings already vitally stained or became stained in the first few minutes after collecting them. They were easily visible to the naked eye and any subsequent manipulation could be carried out without any additional visual aid. The majority of the ova were observed in Ringer's fluid in the living state and were photographed under the Leitz's Panphot on panchromatic plates and on Kodachrome films. Some tubal eggs and also some uterine blastocysts were fixed by adding Heidenhein's Susa fixative drop by drop to the Ringer's solution. Afterwards the eggs were embedded in agar using the technique described by Samuel (1944). In the agar the eggs were dehydrated in slow stages and carried into paraffin wax. Sections, after treating them with iodine-alcohol, were stained in 0·5 per cent. aqu. toluidin solution, dehydrated and enclosed in D.P.X.

DESCRIPTION

Even in the earliest specimens examined in the present series (20 hours after mating) the corona radiata cells were virtually absent, or if occasional clusters of them were left attached to the zona pellucida, they became buried under the gradually thickening albuminous coat. The ultimate fate of these corona radiata cells is still open to speculation. After vital staining the albuminous coat acquired

a distinct reddish colour (γ metachromasia) while the segmenting egg and the zona pellucida itself appeared blue (Plate, fig. 1). Careful observation under suitable lighting conditions revealed a delicate, concentric stratification of the albuminous coat, thus indicating its appositional growth. This stratification is even more obvious on sections of fixed and embedded specimens (Plate, fig. 2).

In many cases occasional villus-like processes could be observed, probably the result of an unequal deposition of the albuminous matter round the ovum while the ovum remained stationary for a short period in one of the culs-de-sac of the tube. It is conceivable that these villus-like processes are partly responsible for Barry's term 'chorion' applied to the albuminous coat.

The examination of the vitally stained specimens suggests that the albuminous matter is deposited by the tubal epithelium and is not a product of the ovum. Occasionally an albuminous coat is deposited on a small cluster of cumulus cells or around the fragments of detached tubal villi. Frequently smaller, and even larger, spherules of albuminous matter were observed without any central cellular core whatsoever (Plate, fig. 4).

Our vitally stained tubal eggs revealed an astonishing frequency of early prenatal death. In one instance 44 hours after mating 8 ova were recovered from the tubes. Only 1 of them appeared to be normal and later sectioning revealed that this ovum was at the 16-cell stage of cleavage. In the remaining 7 metachromatically stained spherules there was an intensive, diffuse basophil colouring reaching almost, but not quite, to the surface. There was no evidence of blastomeres (Plate, fig. 3). Examination of the ovaries revealed the presence of 8 fully formed corpora lutea. On the basis of this evidence one must assume that these ova have died and undergone disintegration at some stage of their development prior to the 16-cell stage.

In all of the specimens recovered from the uterine horns a gradual thinning of the albuminous coat was observed as the blastocysts enlarged. In these expanding blastocysts the more superficial layers of the albuminous coat still show intense metachromasia, but in the deep layers this is replaced by a distinct blue colour which reaches its greatest intensity at, or adjacent to, the zona pellucida. These staining characteristics are most marked in the more advanced blastocysts and these observations seem to indicate that the albuminous coat is undergoing a progressive physico-chemical change in consequence of which it is losing its metachromatic properties.

In a $5\frac{1}{2}$ days' specimen five large blastocysts were recovered from one uterine horn; a corresponding number of corpora lutea were found in the ovary of the same side. In the other horn, which was dissected, only one blastocyst was found, although there were three corpora lutea in the corresponding ovary. This single blastocyst was attached to the antimesometrial side of the uterus and was fixed *in situ* for histological study. The other five blastocysts were vitally stained and revealed under the microscope an intact zona pellucida, but a complete absence of the albuminous coat.

DISCUSSION

'Except for certain notable investigations of ovarian dynamics, there has been no extensive inquiry into the physiology of living mammalian ova. . . . Experimentation has lagged presumably because of the difficulty of handling living ova' (Pincus, 1936). Since these words were written much has been achieved in this comparatively neglected field of embryology. Nevertheless the emphasis is still on the study of non-mammalian eggs and there is a tacit assumption that conditions which obtain in non-mammalian ova would also hold good for mammalian eggs.

The technique used for securing mammalian ova has changed very little since Cruikshank carried out his pioneer experiments in 1797, and, as already stated, until recently few histochemical methods have been used in the study of mammalian eggs. In the present investigation our interest has centred on the albuminous coat of the rabbit ovum, and for this purpose toluidin blue was the obvious vital dye to employ. After the vital staining of the albuminous coat the rabbit ova were, so to speak, enlarged and brought well within the limits of naked-eye visibility. Since these experiments were started some years ago, many other vital dyes have been used while searching for human and other mammalian eggs, and undoubtedly this procedure makes the finding and handling of these ova very much easier.

To base conclusions as to the true nature of the albuminous coat entirely on the results of toluidin blue staining may be open to criticism. Nevertheless, regarding metachromasia obtained after toluidin blue staining, Baker (1950) states: 'The reaction is so precise that it is a reliable histochemical test.' Pearse (1953), though admitting that 'there is a fair controversy about the specificity of metachromatic staining reaction obtained with toluidin blue', comes to the conclusion that 'strong gamma (red) metachromasia appears to indicate presence of acid mucopolysaccharides'. Consequently we feel that at the present stage of our study this simple method—applicable to the living ovum—is satisfactory.

According to Holmgren & Wilander (1937) and also Hess & Hollander (1947) the intensity of metachromasia after toluidin blue staining is a reliable indication of the degree of sulphuration of the molecule. Heparin, a poly-sulphuric acid ester of the polysaccharide group, is most strongly metachromatic, epithelial mucus and chondro-mucoid are both mono-sulphuric acid esters and are optical isomers. Epithelial mucus, however, is noticeably more metachromatic in its staining response to toluidin blue than is chondroitin sulphuric acid, the latter remaining unstained at the concentrations of toluidin blue we have employed in our vital staining experiments.

In view of the above considerations, and also because of the support received from the detailed chemical and histochemical studies of Braden (1952), there seems to be little doubt that the albuminous coat covering the tubal eggs of the rabbit contains considerable quantities of sulphurated mucopolysaccharides of

epithelial origin, and we agree with Braden that there is every justification for the plea that instead of 'albuminous' it should be designated as a 'mucous' or 'mucopolysaccharide' coat.

The enlargement of the blastocyst cavity was described by Assheton (1894), who came to the conclusion that during this period fluid collects in the blastocyst and that it is the increased hydrostatic pressure which is responsible for the enlargement. Heuser & Streeter (1929) are of the opinion that the formation of the segmentation cavity is the result of the precocious differentiation and functional activity of the trophoblast. Pincus (1936) observed that the formation and enlargement of the blastocyst cavity does not take place in rabbit ova cultured on artificial nutritive media, and he more or less postulated the necessity of a uterine factor in the initiation of the expansion. Gregory (1930) observed that while the mucous coat of the tubal egg is viscous and tends to adhere to the bottom of the dish and readily accumulates foreign particles, that surrounding the early uterine blastocyst is less viscous. He believes that the mucus gives up its water to the expanding ovum, or that it is digested and absorbed by the egg, which is probably aided in this process by the uterine secretions.

In our series the vitally stained young uterine blastocysts showed a gradual reduction in the thickness of the mucous coat around the expanding ovum. Furthermore, it was observed that in the deeper layers, adjoining the zona pellucida, there was a distinct loss of metachromasia. The resulting blue staining of this area would suggest an enzymatic hydrolysis and a gradual depolymerization of the mucous coat. The 'spreading response' which accompanies this process should and could provide the necessary hydrodynamic force for the expansion of the blastocyst. The hydrolysis starting in the deepest, juxta-ovular layer of the mucous coat seems to indicate that the enzyme is discharged by the ovum and its production is probably the function of the trophoblast, but on the other hand the observations of Pincus on cultured rabbit ova quoted above strongly suggest the precipitating, or initiating, influence of an additional and so far unknown uterine factor.

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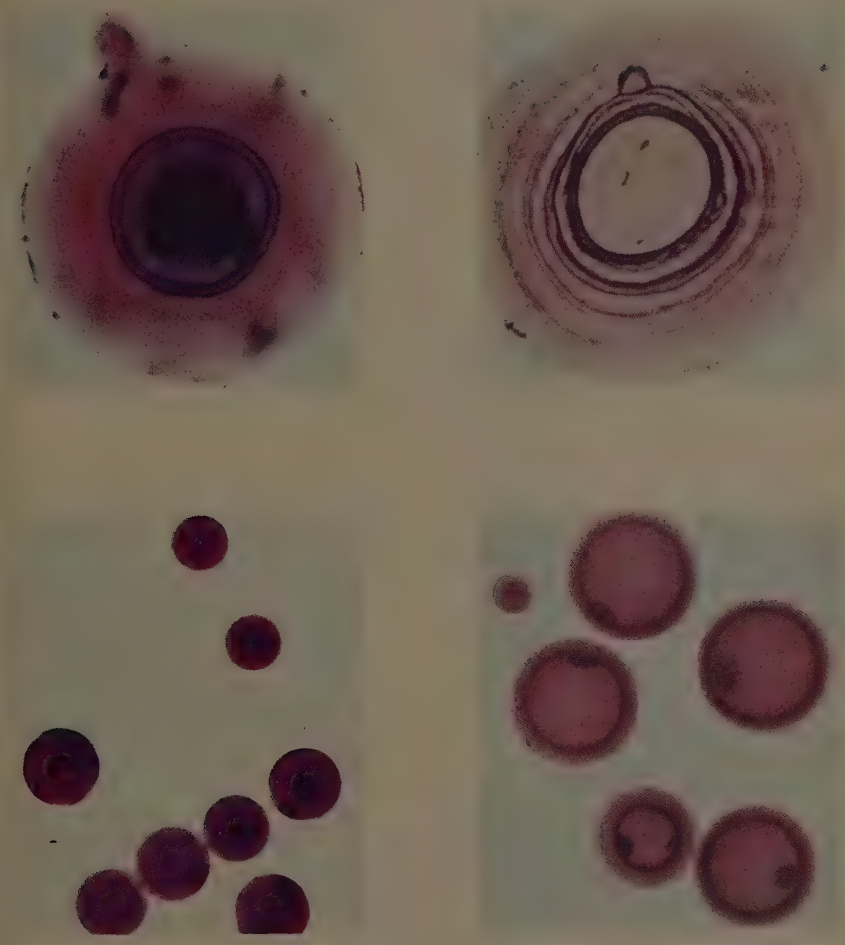
EXPLANATION OF PLATE

FIG. 1. Morula of rabbit 20 hours after mating. Vitally stained with toluidin blue and photographed as a whole mount in Ringer's fluid. Kodachrome.

FIG. 2. The same morula fixed in Susa, embedded by the agar method in paraffin, sectioned and stained with toluidin blue. Kodachrome.

FIG. 3. Rabbit ova recovered from the uterine tube 44 hours after mating and vitally stained with toluidin blue. Observe that only one (at top of picture) is normal; in the others the diffuse basophilia indicates an early prenatal death. Kodachrome.

FIG. 4. Expanding blastocysts recovered from the uterine horns 86 hours after mating, and vitally stained with toluidin blue. Observe the gradual outward disappearance of the metachromasia of the albuminous coat. Kodachrome.



P. BACSICH & W. J. HAMILTON

Plate 1

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